1-1-2012

Mechanisms of acute brain injury following aneurismal subarachnoid hemorrhage: the role of acute microcirculatory failure. An experimental study in mice.

Khalid Abubaker
Royal College of Surgeons in Ireland

Mechanisms of acute brain injury following aneurismal subarachnoid hemorrhage: The role of acute microcirculatory failure. An experimental study in mice

A Thesis Submitted To The Royal College of Surgeons in Ireland for the Degree of Doctor of Medicine.

By

Khalid Abubaker

MBBS, IMRCS

January 2012

Supervisors: Prof. Nikolaus Plesnila & Prof. Ciaran Bolger

This work is carried out in the Laboratory of Experimental Neurosurgery in the Department of Neurodegeneration and Physiology& Molecular Physics in the Royal College of Surgeons in Ireland.

Supervisors:

Scientific supervisor:

Prof. Nikolaus Plesnila
Dept. of Neurodegeneration
Royal college of surgeons in Ireland
123 St. Stephen's Green
Dublin 2.

Clinical Supervisor:

Prof. Ciaran Bolger
Department of Neurosurgery
Beaumont Hospital
Beaumont Road
Beaumont
Dublin 9.

Table of Contents

Dedications

This thesis is especially dedicated to my father Mahmoud Abubaker Mahmoud, who I sadly lost on 19/12/2010.

We will never forget you.

Acknowledgements

I would like to thank my very inspirational supervisor Prof. Plesnila for all his efforts which made this work possible. He was always available when needed to provide guidance, help and support.

My thanks are also to Prof. Bolger, who was the first to encourage me to spend this stage in my career in doing research and continue to be supportive throughout all the stages of this work.

I would also want to thank all the members of Neurodegeneration department: Chen G, Kenney G, Schwarzmaier S, Meissner L, Gallozzi M, McGarry N, Duff S, Osterstock G, Schuler K, De Chaumont C, and Balbi M. I thank them for creating a very strong team spirit and a friendly atmosphere which made working in the laboratory an enjoyable and pleasant experience.

I would also like to acknowledge all the staff in the RCSI main campus including members of the BRF, research office, security and building services and all scientist and researchers in the college for their joint effort to facilitate and encourage research in RCSI.

Communications from this thesis

1- The role of perivascular blood in the etiology of acute microcirculatory failure after experimental subarachnoid hemorrhage.

-An oral presentation at Young Life Scientist Ireland Inaugural scientific meeting. 12/11/2011.

2- Acute microcirculatory vasospasm and microthrombosis early after experimental subarachnoid hemorrhage.

- An oral presentation at the British Neurovascular Group annual meeting 09-10 Feb. 2012.

3- Analysis of the role of extracellular hemoglobin in the etiology of microvasospasm and microthrombosis after experimental subarachnoid hemorrhage.

- An oral presentation at the British Neurovascular Group annual meeting 09-10 Feb. 2012.

Abbreviations

CBF	Cerebral blood flow
CCT	Cerebral circulation time
CPP	Cerebral perfusion pressure
CSF	Cerebrospinal fluid
CT	Computerized tomography
$ETPCO_2$	End tidal PCO_2
ICP	Intracranial pressure
ITSN	Intrathecal sodium nitroprusside
IVM	Intravital microscopy
IVSN	Intraventricular sodium nitroprusside
Hrs	Hours
Min	Minute
MABP	Mean arterial blood pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
$PbrO_2$	Brain tissue partial pressure of oxygen
PET	Positron emission tomography
RBCs	Red blood cells
SAH	Subarachnoid hemorrhage
SEM	Standard error of measurement
SNP	Sodium nitroprusside
STD	Standard deviation

Summary

Background: Aneurismal subarachnoid hemorrhage is the most devastating type of stroke, with two thirds of the affected individuals either die or remain with moderate to severe disability. The majority of patients die during the first few days after subarachnoid hemorrhage (SAH) (Macpherson, Lewsey et al. 2011). This mortality is most likely attributed to a severe, cerebral perfusion pressure-independent reduction of cerebral blood flow (CBF). The mechanisms of this CBF decrease are largely unknown and may be related to disturbances at the level of the cerebral microcirculation. This project is an attempt to study the pathophysiological mechanisms by which subarachnoid hemorrhage leads to cerebral perfusion pressure- independent cerebral ischemia. We examined the most widely accepted theory that oxyhemoglobin induced nitric oxide depletion plays a key role in the etiology of cerebral ischemia and brain injury after subarachnoid hemorrhage. In order to achieve this, we investigated any role intravascular free hemoglobin might play in causing nitrogen oxide depletion by measuring plasma hemoglobin levels after experimental subarachnoid hemorrhage and comparing it with normal animals. We then studied cerebral microcirculation at 1-3 hours after experimental subarachnoid hemorrhage to check for any ischemic changes at this stage after experimental and, also, to test the correlation between perivascular blood, which served as an index for perivascular hemoglobin, and these ischemic changes.

Methods: Subarachnoid hemorrhage was induced in C57/BL6 mice using Circle of Willis endovascular perforation technique (Feiler, Friedrich et al. 2010). Intravital fluorescence microscopy was used to study cerebral microcirculation1-3 hours after experimental subarachnoid hemorrhage. Plasma hemoglobin was measured using the spectrophotometric scanning method of Blakney and Dinwoodie.

Results: Animals had normal physiological parameters throughout the experimental period. Post-hemorrhagic intracranial pressure (ICP) rose to 91 +/- 29 mmHg, indicating a comparable severity of SAH in all animals. 56% of observed cerebral vessels were affected by microvasospasm and microthrombosis. These changes were mainly seen in capillaries and small arterioles. 39% of all vasospasms were detected in vessels with 10-20 µm caliber, 28% of vasospasms occurred in vessels with 20-30 µm caliber and 10% in blood vessels with 30-40 µm in diameter. 59% of microthrombi were seen blood vessels <10 µm in diameter and 37% of them occur in vessels with 10-20 µm calibers. 93% of all microvasospasm are associated with blood in the perivascular space compared to only 7% of vasospasm occurring in the absence of perivascular blood. 79% of microthrombi were observed in vessels coated with perivascular blood, compared to 21% of microthrombi detected in the absence of perivascular blood. Plasma hemoglobin levels at 3 & 24 hours after experimental subarachnoid hemorrhage were similar to levels of plasma hemoglobin in control animals. 72 hours after SAH, the levels are higher in SAH animals but the difference is not statistically significant.

Conclusions: As early as 1 hour after subarachnoid hemorrhage there are widespread microvasospasms and microthrombosis affecting cerebral microcirculation. These changes are severe enough and extensive enough to cause early post hemorrhagic cerebral ischemia and to serve as an explanation for early mortality following SAH. Extra- rather than intra-vascular hemoglobin is most likely responsible for cerebral microvasospasm and microthrombosis after experimental subarachnoid hemorrhage.

Chapter 1

INTRODUCTION

When persons in good health are suddenly seized with pains in the head, and straightway are laid down speechless, and breathe with stertor, they die in seven days.

Hippocrates 460-370 BC, *Aphorisms on Apoplexy*

1.1. Aneurismal subarachnoid hemorrhage

Aneurismal subarachnoid hemorrhage (SAH) is a type of hemorrhagic stroke caused by the rupture of an intracranial aneurysm. It accounts for 75-85% of all cases of spontaneous (non-traumatic) subarachnoid hemorrhage(van Gijn J Fau - Kerr, Kerr Rs Fau - Rinkel et al.). Its estimated annual rate in most western populations is 6-10 per 100,000 (Linn, Rinkel et al. 1996; Forget, Benitez et al. 2001; Seibert, Tummala et al. 2011). Aneurismal subarachnoid hemorrhage also represents 15% of all intracranial hemorrhages and about 5% of all strokes (Pluta 2005; Macpherson, Lewsey et al. 2011). It has unique pathophysiological characteristics that differentiate it from other forms of intracranial hemorrhage (epidural, subdural, intraventricular and intracerebral hemorrhages). First, the source of the bleeding in aneurismal subarachnoid hemorrhage is usually one of the major intracranial arteries, which will, therefore, result in a high pressure/ high volume hemorrhage that can cause serious brain damage. Second, the clots that result from subarachnoid hemorrhage are poorly localized and spread throughout the subarachnoid space and remain there for days covering blood vessels (Zhang Zd Fau - Yamini, Yamini B Fau - Komuro et al.; Pluta 2005).

The presence of blood in direct contact with cerebral blood vessels will precipitate alterations in the physiological forces that regulate the vascular tone leading to widespread vasospasm, which will result in reduced cerebral blood flow and cerebral ischemia (Zhang Zd Fau - Yamini, Yamini B Fau - Komuro et al.).

1.2. Cerebral aneurysms

Cerebral aneurysms are detected in 2-6% of the population (Wardlaw and White 2000; White and Wardlaw 2003; Pluta 2005; Seibert, Tummala et al. 2011). The percentage of ruptured: unruptured aneurysm is 1:1, i.e. 50% of these aneurysms rupture (Greenberg 2006). The most frequent and most serious presentation of ruptured intracranial aneurysms is major subarachnoid hemorrhage (Seibert, Tummala et al. 2011). Aneurismal subarachnoid hemorrhage is often accompanied by other pathologies such as intracerebral hemorrhage (19-40%) (Tokuda, Inagawa et al. 1995; Kopera, Majchrzak et al. 1999) , intraventricular hemorrhage (13-28%) (Greenberg 2006) and acute hydrocephalus (20%) (Hasan, Lindsay et al. 1991; Suarez-Rivera 1998). Coexistence of any of these pathologies along with aneurismal subarachnoid hemorrhage adversely affects the prognosis and adds to the already high mortality and morbidity in these patients (Hauerberg J Fau - Eskesen, Eskesen V Fau - Rosenorn et al. ; Nakagawa T Fau - Suga, Suga S Fau - Mayanagi et al. ; Mohr, Ferguson et al. 1983; Hasan, Lindsay et al. 1991; Suarez-Rivera 1998; Mayfrank, Hutter et al. 2001; Rosengart, Schultheiss et al. 2007).

The exact pathophysiological mechanisms responsible for the formation of cerebral aneurysms are not fully understood. Cerebral arteries have certain characteristics that make them susceptible to the development of aneurysms. In contrast to extracranial blood vessels, cerebral arteries have less elastic tissue in the tunica media and adventitia, a thinner adventitial layer and lesser muscle mass in the tunica media (Wilkinson 1972; Greenberg 2006). In addition, cerebral blood vessels lie within the subarachnoid space, and that is why they have very little supporting connective tissue (Greenberg 2006). Most aneurysms tend to develop at the bifurcations of cerebral arteries, where there is irregularity in the collagen matrix in the arterial wall and there is maximum hemodynamic stress generated by alterations of the radii of blood vessels at these bifurcations (Foutrakis, Yonas et al. 1999; Turner, Tebbs et al. 2001). This predilection of aneurysms for the bifurcation sites stresses the importance of hemodynamic stresses (Roach, Scott et al. 1972; Foutrakis, Yonas et al. 1999; Turner, Tebbs et al. 2001; Nixon, Gunel et al. 2010; Cebral, Mut et al. 2011; Cebral, Mut et al. 2011) and structural alteration of the arterial wall (Scanarini, Mingrino et al. 1978; Kim, Cervos-Navarro et al. 1993; Kulcsar, Ugron et al. 2011) in the development of cerebral aneurysms. Congenital as well as acquired factors combine to mediate the formation, growth and rupture of cerebral aneurysms. It's believed that certain individuals have genetic predisposition to the development of cerebral aneurysms due to inherited malformations of the arterial walls, such as defects in reticular fibers in the medial layer (Hegedus ; Ostergaard, Reske-Nielsen et al. 1989; Chyatte, Reilly et al. 1990; Ostergaard 1991) or disruption of

the internal elastic lamina (Yong-Zhong and van Alphen 1990; Kim, Cervos-Navarro et al. 1993). Acquired factors such as atherosclerosis (de la Monte, Moore et al. 1985), hypertension (Franks 1978; de la Monte, Moore et al. 1985)and smoking (Weir Bk Fau - Kongable, Kongable Gl Fau - Kassell et al. ; Rinkel, Djibuti et al. 1998; Juvela, Porras et al. 2008) also play an important role in the etiology of cerebral aneurysms, probably in the setting of an existing congenital predisposition (Pluta 2005; Greenberg 2006). The most common type of intracranial aneurysms is saccular aneurysms or berry aneurysms. These are usually located on major intracranial arteries at branching or bifurcating points along these arteries (Ferguson 1972; Foutrakis, Yonas et al. 1999; Kulcsar, Ugron et al. 2011). About 85-90% of berry aneurysms occur in the carotid system in one of the arteries forming the Circle of Willis at the base of the brain (internal carotid artery, middle cerebral artery, anterior cerebral artery, anterior communicating and posterior communicating) (Okuyama T Fau - Sasamori, Sasamori Y Fau - Takahashi et al. ; Greenberg 2006), and 5-15% occur in the vertebrobasilar system, predominantly in one of the following three arteries: vertebral artery, basilar artery and the posterior inferior cerebellar artery (Greenberg 2006).

1.3. Mortality & morbidity from aneurismal subarachnoid hemorrhage

Aneurismal subarachnoid hemorrhage is regarded as the most fatal type of stroke. About 16% of individuals who suffer aneurismal subarachnoid hemorrhage die before reaching medical care (Pobereskin 2001; Macpherson, Lewsey et al. 2011). Despite advances in treatment, that led to decline in mortality rates over the past three decades (Hop, Rinkel et al. 1997; Nieuwkamp, Setz et al. 2009; Macpherson, Lewsey et al. 2011), 28% of patients still die within 30 days of the onset of subarachnoid hemorrhage (Nieuwkamp, Setz et al. 2009). Among those who survive 30% remain with moderate to severe disability(Hop, Rinkel et al. 1997). Overall, Two thirds of patients with aneurismal subarachnoid hemorrhage will either die or end with moderate to severe disability (Seibert, Tummala et al. 2011). Around 21% of all mortalities occur during the first 24 hours (Pobereskin 2001), suggesting that the acute brain injury from the initial hemorrhage is responsible for the greatest proportion of the mortalities. Another important factor which is responsible for this high morbidity and mortality is re-bleeding, which accounts for 15-20% of all mortalities. Re-bleeding and acute hydrocephalus are the only two major mortality factors that can be prevented or treated successfully. Delayed cerebral vasospasm, or angiographic vasospasm, which occurs 72 hours after the hemorrhage and affect large cerebral arteries, is considered to be the cause of death in 7% of patients and to cause neurological deficit in another 7%(Greenberg 2006). Hence, the phenomenon of delayed cerebral vasospasm has been the focus of extensive experimental and clinical research, looking for effective therapies to tackle vasospasm, and reduce the inci-

dence of cerebral ischemia and delayed ischemic neurological deficit. Over the years, there has been a reduction in the incidence of delayed cerebral vasospasm, by itself, but that did not reduce the incidence of cerebral ischemia or improve the long term outcome (Macdonald, Pluta et al. 2007; Beck and Raabe 2011). In fact, delayed ischemic injury and poor outcome are not always associated with delayed cerebral vasospasm (Vergouwen, Vermeulen et al. 2008). 21% of SAH survivors, who do not have delayed vasospasm, develop delayed ischemic injury (Alaraj, Charbel et al. 2009; Sehba, Pluta et al. 2011), and only 20-30% of those, who do develop delayed vasospasm, actually, suffer from delayed ischemic injury (Alaraj, Charbel et al. 2009; Sehba, Pluta et al. 2011). In addition, therapies that successfully treat angiographic vasospasm failed to improve the clinical outcome in patients with aneurismal subarachnoid hemorrhage. The latest example is the findings from the three phases of the endothelial receptor antagonist, clazosentan, trial in patients with aneurismal subarachnoid hemorrhage, which showed that although the drug reduced the incidence of vasospasm, it did not improve the clinical outcome (Vajkoczy, Meyer et al. 2005; Macdonald, Kassell et al. 2008; Macdonald, Higashida et al. 2011). Similar results were obtained with transluminal balloon angioplasty, which was effective against angiographic cerebral vasospasm, but failed to improve the long term clinical outcome (Coenen, Hansen et al. 1998; Polin, Coenen et al. 2000; Murai, Kominami et al. 2005). On the other hand, there are other therapies which improve the clinical outcome without showing any effect on angiographic vasospasm. The use of calcium channel antagonist nimodipine, for example, has

beneficial effects on the outcome of patients with aneurismal subarachnoid hemorrhage without preventing angiographic vasospasm (Philippon, Grob et al. 1986; Jan, Buchheit et al. 1988; Petruk, West et al. 1988; Feigin, Rinkel et al. 1998). More importantly, 75% of all mortalities occur within 72 hours after the subarachnoid hemorrhage (Pobereskin 2001). Therefore, it's evident from the outcome statistics that the mortality rates are highest during the first 24- 72 hours after the hemorrhage. This indicates that the pathophysiological events that take place within this early period after the hemorrhage determine the prognosis of these patients. Because of the observations that the mortality rates are highest during the first 72 hours, and the persistence of ischemic brain injury and poor outcome irrespective of delayed cerebral vasospasm, the focus of experimental and clinical research has shifted towards studying the mechanisms that lead to the early brain injury that occurs during the first 72 hours following subarachnoid hemorrhage.

1.4. Early brain injury after subarachnoid hemorrhage

The mechanisms of early brain injury after aneurismal subarachnoid hemorrhage are not fully understood. The two main events that are produced by subarachnoid hemorrhage and can potentially cause early brain damage are the initial burst of blood at the time of aneurysm rupture and the presence of blood within the subarachnoid space afterwards. Each of these two events evokes a sequence of pathophysiological responses that can result in early brain injury. The release of blood from the ruptured aneurysm triggers a series of changes in

the hemodynamics of the cerebral circulation. These changes have been extensively studied in experimental and clinical studies, and the results of these studies have led to important conclusions in the understanding of the mechanisms of early brain injury after subarachnoid hemorrhage. A summary of the cerebral hemodynamic changes caused by initial bleeding can be drawn from these studies. The sudden burst of blood from the ruptured aneurism causes an immediate and sharp rise in intracranial pressure (ICP) within seconds (Heuer Gg Fau - Smith, Smith Mj Fau - Elliott et al. ; Westermaier T Fau - Jauss, Jauss A Fau - Eriskat et al. ; Bederson, Germano et al. 1995; Torok, Klopotowski et al. 2009; Feiler, Friedrich et al. 2010; Schubert, Seiz et al. 2011). After reaching the peak, ICP gradually decreases within minutes, reaching a plateau at figures that are above its baseline values (Bederson, Germano et al. 1995; Feiler, Friedrich et al. 2010). Concomitant to the initial surge in ICP after SAH, cerebral perfusion pressure (CPP) falls sharply (Bederson, Germano et al. 1995; Prunell, Mathiesen et al. 2003; Feiler, Friedrich et al. 2010), reaching its lowest value within 60 seconds after subarachnoid hemorrhage (Bederson, Germano et al. 1995). Cerebral blood flow (CBF) follows CPP during this period and drops sharply (Westermaier T Fau - Jauss, Jauss A Fau - Eriskat et al. ; Bederson, Germano et al. 1995). After reaching its lowest value (within a minute of the onset of SAH), CPP starts to recover and gradually increases towards the baseline, ultimately reaching values that are above cerebral autoregulation threshold. However, CBF continues to decrease and reaches its lowest value at a time when CPP is increasing towards baseline (Westermaier T Fau - Jauss, Jauss A

Fau - Eriskat et al.; Bederson, Germano et al. 1995). Ultimately, by 60 minutes after SAH, CBF remains 50-80% below its baseline values despite CPP returning to values that are normally sufficient to maintain normal CBF (around 50mmHg) (Bederson, Germano et al. 1995; Prunell, Mathiesen et al. 2003; Feiler, Friedrich et al. 2010). These findings are further augmented by similar observations in clinical studies. Schubert et al. observed a persistent and severe reduction of cerebral blood flow in patients during the first 12 hours after SAH despite the presence of normal ICP and CPP (Schubert, Seiz et al. 2009). Thus, it's evident that factors other than CPP are important contributors to the acutely decreased cortical blood flow after SAH. Primary reduction of cerebral metabolic activity, and acute SAH-induced vasoconstriction are two potential mechanisms proposed to be responsible for the CPP independent reduction of cortical blood flow following SAH (Bederson, Germano et al. 1995; Nieuwkamp, Setz et al. 2009; Westermaier, Jauss et al. 2009). Carpenter et al. reported a reduced regional cerebral oxygen metabolism on positron emission tomography (PET) scans of patients in the early stages after aneurismal subarachnoid hemorrhage without evidence of angiographic vasospasm (Carpenter, Grubb et al. 1991). However, experimental studies examining cerebral blood flow and cerebral metabolic activity simultaneously have found that the decrease in cerebral blood flow precedes the decrease of cerebral metabolic activity, indicating that reduction in cerebral metabolic activity is not responsible for reduced CBF (Bederson, Germano et al. 1995). Hence, it's concluded that acute vasoconstriction, most probably at the microcirculatory level, is the most likely mechanism leading to

the CPP independent reduction in CBF which is seen in the early stages after subarachnoid hemorrhage (Schubert, Seiz et al. 2009). The significance of microcirculatory vasospasm as a cause of acute hypoperfusion after SAH is highlighted by the dissociation between the presence of reduced cerebral perfusion, and angiographic evidence of vasospasm, a phenomenon that has been described in many clinical studies (Jakobsen M Fau - Overgaard, Overgaard J Fau - Marcussen et al. ; Grubb, Raichle et al. 1977; Kelly, Gorten et al. 1977). From these studies, collectively, we can see that there is enough clinical and experimental evidence to suggest acute microcirculatory vasospasm contributes directly to ischemic brain injury and poor outcome following subarachnoid hemorrhage (Bederson Jb Fau - Levy, Levy Al Fau - Ding et al. ; Sehba, Ding et al. 1999). Although the ICP does rise to ischemic levels as the blood bursts from the ruptured aneurysm, this is unlikely to contribute significantly to early brain damage because these ischemic ICP levels last only for few minutes.

1.5. Mechanisms of acute microcirculatory vasoconstriction

The exact mechanisms mediating acute vasoconstriction after subarachnoid hemorrhage are not entirely clear. The subarachnoid hemorrhage results in thick subarachnoid blood clots coating the adventitial surfaces of cerebral arteries (Findlay, Macdonald et al. 1991). It is well known that the distribution and severity of post hemorrhagic vasospasm correlates well with the volume and distribution of the subarachnoid blood clots on the computerized tomography (CT) scans of patients with aneurismal subarachnoid hemorrhage (Fisher, Kistler et

al. 1980; Kistler, Crowell et al. 1983; Findlay, Macdonald et al. 1991). The exact element of blood that causes vasospasm has been the subject of intense research. The whole blood by itself is well known to have a definite spasmodic action on cerebral arteries (Echlin 1965; Zervas, Kuwayama et al. 1973; White, Hagen et al. 1975). However, the vasoconstrictor action of the subarachnoid hematoma is attributed to the red blood cells (RBCs) component of the hematoma (Duff, Louie et al. 1988; Mayberg, Okada et al. 1990; Nozaki, Okamoto et al. 1990; Peterson, Roussos et al. 1990; Yamamoto, Clower et al. 1991). After subarachnoid hemorrhage, the RBCs remain in the subarachnoid space for days, undergoing a process of slow hemolysis (Mayberg, Okada et al. 1990; Yamamoto, Clower et al. 1991). This process of hemolysis is essential for the for the spasmogenic action of the RBCs, because intact RBCs does not constrict cerebral arteries (Macdonald and Weir 1991). The result of RBCs hemolysis is the release of hemoglobin and its metabolites from the RBCs into the perivascular space. Of all the hemoglobin metabolites, oxyhemoglobin has been identified as the principal spasmogen responsible for cerebral vasospasm after subarachnoid hemorrhage (Asano ; Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al. ; Macdonald, Weir et al. 1991; Pluta, Oldfield et al. 1997; Pluta, Afshar et al. 1998). Oxyhemoglobin has several mechanisms of action that can explain its role in the etiology of post hemorrhagic cerebral vasospasm. Particularly important action of oxyhemoglobin, which attracted huge interest, is its ability to cause depletion of nitric oxide (NO) from the vascular wall. Oxyhemoglobin is a powerful scavenger of NO due to the very high affinity between NO and the

heme moiety of the hemoglobin molecule, (Goretski and Hollocher 1988; Ignarro 1990) . It is hypothesized that the NO scavenging effect by oxyhemoglobin is compounded by decreased production of NO, which is caused by oxyhemoglobin induced inhibition of both endothelial (Jung Cs Fau - Iuliano, Iuliano Ba Fau - Harvey-White et al.) and neuronal (Pluta, Thompson et al. 1996) nitric oxide synthase (NOS), and these two actions will, ultimately, result in depletion of NO from the vascular wall. It's known that NO is an important autoregulatory molecule in the cerebral circulation with many physiological roles including activation of soluble guanylate cyclases, which increase production of cyclic guanosine monophosphate leading to the relaxation of the vascular smooth muscles and vasodilatation (Guix, Uribesalgo et al. 2005). The depletion of nitric oxide from the vessel wall will, therefore, remove its vasodilatory effects, leading to vasospasm under the influence of unopposed vasoconstrictor mechanisms such as endothelin-1.

The effects of acute post hemorrhagic microcirculatory vasospasm on cerebral perfusion are compounded by the presence of wide spread microthrombosis across cerebral microcirculation in the early stages after subarachnoid hemorrhage. These wide spread microthrombi have been demonstrated in experimental studies within minutes of subarachnoid hemorrhage (Sehba, Mostafa et al. 2005). They have also been demonstrated on transcranial Doppler ultrasound studies of patients with aneurismal subarachnoid hemorrhage (Romano, Forteza et al. 2002). Similar to vasospasm, the amount and distribution of microthrombi is shown to strongly correlate with the volume and distribution of

the subarachnoid blood (Stein, Browne et al. 2006) Moreover, microvasospasm and microthrombi predominately occur in the same vessels in close proximity to one another, with microthrombi typically occupying the proximal ends of microvasospasms (Friedrich, Flores et al. 2010; Friedrich, Muller et al. 2011). This suggests a common factor is, perhaps, responsible for the etiology of both. Depletion of NO from the vascular wall, which is regarded as the most likely mechanism responsible for post-hemorrhagic vasospasm (Pluta, Dejam et al. 2005), causes platelet aggregation and thrombus formation by removing the inhibitory effects of NO on platelets aggregation (Friedrich, Flores et al. 2010). Thus, it's generally believed that, NO depletion, induced by oxyhemoglobin released from the hemolytic break down of the RBCs within the subarachnoid blood clot, mediates the formation of microcirculatory vasospasm and microthrombosis, leading to microcirculatory dysfunction and CPP independent acute cerebral hypoperfusion, which will ultimately result in ischemic brain injury in patients with aneurismal subarachnoid hemorrhage.

1.6. The aim of the current project

One of the main pillars of the hypothesis of hemoglobin induced NO depletion is the cause of post- hemorrhagic cerebral vasospasm and microthrombosis, is the inhibition of endothelial NOS by oxyhemoglobin. In order to test this hypothesis, we measured the level of free hemoglobin in the plasma of mice subjected to experimental SAH, and compared it to that of healthy mice. Our results showed there is no increase in plasma hemoglobin at 3 hours and 24 hours after experi-

mental SAH in mice. We found an increase in the plasma hemoglobin level of mice at 72 hours after experimental SAH, but the increase was not statistically significant. These findings practically rolled out direct inhibition of endothelial NOS by plasma hemoglobin as a mechanism involved in NO depletion that will subsequently result in early post hemorrhagic cerebral vasospasm after SAH.

The second part of this project was an intravital microscopy (IVM) study of cerebral microcirculation within 1-3 hours after experimental subarachnoid hemorrhage in mice. This time window is chosen because our ultimate aim is to study the cerebral microcirculation as close to the time of the hemorrhage as possible. In a study conducted in our laboratory in Munich, Friedrich et al. demonstrated the presence of microcirculatory changes up until three hours after subarachnoid hemorrhage (Friedrich, Muller et al. 2011).

Therefore, the two main objectives of the intravital microscopy study are:

1- To investigate the presence of microvasospasm and microthrombi within the cerebral microcirculation at the very early stages after SAH.

2- To investigate the role of perivascular hemoglobin in the etiology of NO depletion that is proposed to subsequently result in post- hemorrhagic vasospasm. This was achieved, indirectly, by studying the degree of correlation between the presence of microvasospasms and microthrombi within the blood vessels and the presence of perivascular blood in the respective vessels.

The findings of the study confirmed the presence of ischemic changes in form of microvasospasms and microthrombosis affecting about half of the blood vessel

segments investigated during the interval of 1-3 hours after experimental SAH. We also found a very strong correlation between the distribution of these ischemic changes and the presence of perivascular blood; which suggests an important role for perivascular hemoglobin, present in the perivascular hematoma, in causing depletion of NO from the perivascular wall, which, subsequently, leads to post- hemorrhagic cerebral ischemia.

CHAPTER 2

MATERIAL & METHODS

2.1. Animals

Male C57B16 mice (body weight of 20 to 26 g) were used for the current project. All animals were obtained from Harlan UK (Shaw's Farm, Bichester, England), and cared for at all stages in accordance with the principals of European Communities Council directive (86/609/EEC). All procedures and experiments in this project were approved by the research ethics committee of the Royal College of Surgeons in Ireland.

2.2. Anesthesia and monitoring

The animal had free access to food and water prior to surgery. General anesthesia was induced by inhalation of 5% isoflurane for 60 seconds followed by intraperitoneal injection of a combination of midazolam (5 mg/kg body weight; Roche Products Limited, Welwyn Garden City, United Kingdom), fentanyl (0.05 mg/kg; Antigen pharmaceuticals, Co. Tipperary, Ireland), and medetomidin (0.5 mg/kg; CP-Pharma Handelsges. MbH, Burgdorf. Germany). For procedures that require more than an hour, an additional intraperitoneal injection of a quarter of the induction dose was given to maintain anesthesia. After anesthesia was successfully induced, the mice were orotracheally intubated and mechanically ventilated as previously described (Thai and Plesnila 2007). Briefly, the mice were placed on a 45° angled table, and intubated under direct vision using surgical microscopes. The endotracheal tube used for intubation was fashioned from a commercially available intravenous catheter by shortening the catheter tube to

about 22 mm and its plastic connector to 4 mm. A small hole is also drilled at the tail end of the plastic connector in order to fit the sample tubing which will be connected to the microcapnometer (**See fig. 1**). The mice were then mechanically ventilated by connecting the orotracheal tube, through a Y connector, to a mechanical ventilator (Minivent, Hugo Sachs, Hungstetten, Germany). A controlled mixture of 35-40% oxygen in room air was used for ventilation. End tidal PCO2 was continuously measured by a microcapnometer (C1240, Columbus instruments, Columbus, USA) and maintained at 30 to 35 mmHg by adjusting the ventilation. Body temperature was maintained at 37°C by a thermostatically regulated feed-back controlled heating pad (FHC, Bowdoinham, USA). After completion of the planned experiment and animals were planned for further longer term experiments, anesthesia was terminated by intraperitoneal injection of a combination of atipamezol (2.5 mg/kg; CP-Pharma Handelsges. MbH, Burgdorf. Germany), nalaxone (1.2mg/kg; Antigen pharmaceuticals, Co. Tipperary, Ireland) and flumazenil (0.5 mg/kg; Roche Products Limited, Welwyn Garden City, United Kingdom). Immediately after termination of anesthesia, the endotracheal tube was removed and animals were kept in an incubator at 33°C.

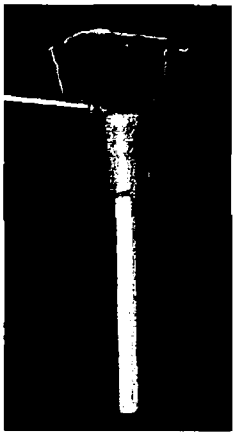

Fig.1. The endotracheal tube. Note the metal tube that connects the endotracheal tube to the microcapnometer to measure ETPCO2.

2.3. Surgical Induction of subarachnoid hemorrhage

SAH was induced in mice using the circle of Willis endovascular perforation model that was first described in rats (Bederson, Germano et al. 1995) and mice (Kamii, Kato et al. 1999; McGirt, Parra et al. 2002; Gao, Wang et al. 2006) and further characterized by Feiler and colleagues (Feiler, Friedrich et al. 2010). For description purposes, the procedure is explained in its consequential steps, by describing first the insertion of the intracranial pressure monitoring device, followed by description of the vascular procedure leading to subarachnoid hemorrhage.

2.4. Intracranial pressure monitoring

After the animals were anaesthetized and the ventilation established, a small skin incision was made over the right temporoparietal area of the skull. The tem-

poralis muscle was then incised and detached from its upper attachment and displaced inferiorly exposing the underlying bone. A small craniotomy was drilled at the junction between the upper and lateral surfaces of the skull and a 5 mm intracranial pressure monitoring probe (Samba Preclin, Samba sensors, Västra Frölunda, Sweden) was inserted into the epidural space for measuring of the intracranial pressure. Semi liquid bone cement was then applied over the craniotomy in order to firmly seal the craniotomy and, at the same time, fix the ICP probe to the skull. The probe was then connected to an intracranial pressure measurement device (Samba Preclin, Samba sensors, Västra Frölunda, Sweden) for continuous monitoring of intracranial pressure.

2.5. Induction of subarachnoid hemorrhage

After placing the intracranial monitor, the animals were then placed in prone position and a midline neck incision was made to expose the left carotid artery and its two terminal branches. A thin prolene suture (Ethicon, San Lorenzo, Puerto Rico) was passed through the external carotid artery into the internal carotid artery and advanced intracranially. Successful induction of SAH was marked by a sudden and sharp rise in intracranial pressure. As the ICP start to rise, the monofilament was gently withdrawn through the external carotid artery and the latter tightly ligated.

2.6. Measurement of free plasma hemoglobin

Free plasma hemoglobin was measured in 4 different groups of animals: the control group, healthy animals, (n = 5), 3 hours after SAH (n = 5), 24 hours after SAH (n= 6) and 72 hours following SAH (n = 6).

The spectrophotometric scanning method of Blakney and Dinwoodie (Kahn, Watkins et al. 1981) was used for determination of plasma hemoglobin.

2.7. Collection of plasma samples

At the desired time point animals were anaesthetized as described previously. With animals in supine position, a coronal incision was made just below the thoracic cage. The peritoneum was opened and the diaphragm clearly identified. A small incision was made across the right part of the diaphragm and gently extended onto its left side, opening the thoracic cavity from its inferior part. Care was taken to avoid excessive bleeding which could potentially lower the total blood volume. The heart was gently positioned so that the right ventricle is clearly seen as the most anterior part of the heart. Using a 23 G needle attached to a 1 ml syringe, about 450-600 µl of blood was very gently aspirated from the right ventricle. In order to avoid in vitro hemolysis at this stage, the ventricle was gently cannulated to avoid excessive tissue trauma, and the withdrawal of blood was done gently to avoid any turbulence during the blood flow into the syringe.

The needle was then removed from the syringe, and the blood was gently transferred from the syringe into an Eppendorf tube. Both the syringe and the Eppendorf tube were pre-coated with heparin in order to avoid clotting of the blood sample, which can also cause in vitro hemolysis of the RBCs. The plasma was separated by centrifuging the blood in the Eppendorf tube, at a speed of 1000 cycles/min for 10 minutes initially, then at a speed of 1600 cycles/min for 20 minutes.

2.8. Procedure and calculation

About 50-100 µl of plasma was withdrawn from the supernatant solution, and diluted to 1000 µi using distilled water. Another solution of 1000 µi of distilled water was prepared to serve as control in order to set the absorbance to zero each time the absorbance of the plasma solution was measured. Each of the two solutions was placed in a separate chamber in the spectrophotometer. The absorbance of the plasma solution was measured three times at each of the wave lengths of 562, 578 and 598 nm. The mean values of these measurements were used to calculate the concentration of hemoglobin from the following equation: 155 x A578-81 x A562-69 x A598 (Kahn, Watkins et al. 1981). The figures of 155, 81, and 69 are the calibration factors for oxyhemoglobin, methemoglobin and bilirubin respectively, and the (A) stands for the mean absorbance values at the corresponding wave length (Kahn, Watkins et al. 1981). The result of this calculation was then multiplied by the factor by which the initial plasma volume

was diluted to 1000 µl. This final result represents the concentration of hemoglobin in the plasma sample in mg/dl.

2.9. Intravital microscopy

Intravital microscopy was conducted using the model established in our laboratory in Munich (Kataoka, Kim et al. 2004; Schwarzmaier, Kim et al. 2010; Friedrich, Muller et al. 2011). After anesthesia and ventilation were established, animals were placed in a stereotactic frame (Stoelting Wood Dale, Illinois. U.S.A). A rectangular 15 × 15 mm cranial window was made over the left parietal cortex. The Dura mater was left intact to preserve the anatomy of the subarachnoid space. The window extends in the coronal plane from the attachment of the temporalis muscle to a point just lateral to the sagittal suture. In the sagittal plane, the window extends from just behind the coronal suture to a point just in front of the occipital suture. A glass cover was placed over the window and firmly fixed to the skull bone surrounding the window using bone cement. The purpose of the glass cover was to prevent brain herniation which will otherwise take place secondary to brain edema that occurs early after subarachnoid hemorrhage.

After the cranial window was done, with the mouse still in the same position, the intracranial pressure monitor was inserted as prescribed previously.

The mouse was then placed in supine position, and a femoral catheter was inserted in the left femoral artery. Subsequently, subarachnoid hemorrhage was induced as prescribed above.

In sham operated animals, the same procedures were performed without inducing subarachnoid hemorrhage, i.e. the monofilament was advanced into the internal carotid artery intracranially, but withdrawn before causing any change in base line ICP.

Once all the surgical procedures were completed, animals were transferred onto a computer controlled microscope stage for analysis of cerebral vasculature. Continuous monitoring of all physiological parameters (body temperature, ETPCO2, MABP) was maintained throughout the duration of intravital microscopy, and arterial blood gas was measured at the end of the experiment.

Visualization of cerebral vasculature was achieved by intra-arterial injection of 0.15 ml of fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular weight 150,000; Sigma-Aldrich, Wicklow. Ireland). The vascular tree of the ipsilateral middle cerebral artery, from arteriolar (10 - 80 µm) to capillary level (< 10 µm), was studied by obtaining repeated short videos along vessel segments and subsequently analyzing the video images post-hoc.

2.10. Statistical analysis

Statistical analysis was done using SigmaStat 11. Plasma hemoglobin results were analyzed using one way analysis of variance (ANOVA). Data on the correlation between microthrombosis and microvasospasm with perivascular blood were analyzed using Mann-Whitney Rank Sum test. Statistical significance was

assumed at P <0.05. Data were presented as mean+/- SEM if not otherwise indicated.

CHAPTER 3

RESULTS

3.1. Plasma hemoglobin levels

Our data showed that plasma hemoglobin levels at 3 and 24 hours after experimental subarachnoid hemorrhage are similar to the levels of plasma hemoglobin in control animals. At 72 hours after SAH, the levels are higher in SAH animals than control animals, but the difference is not statistically significant (**Fig. 2**).

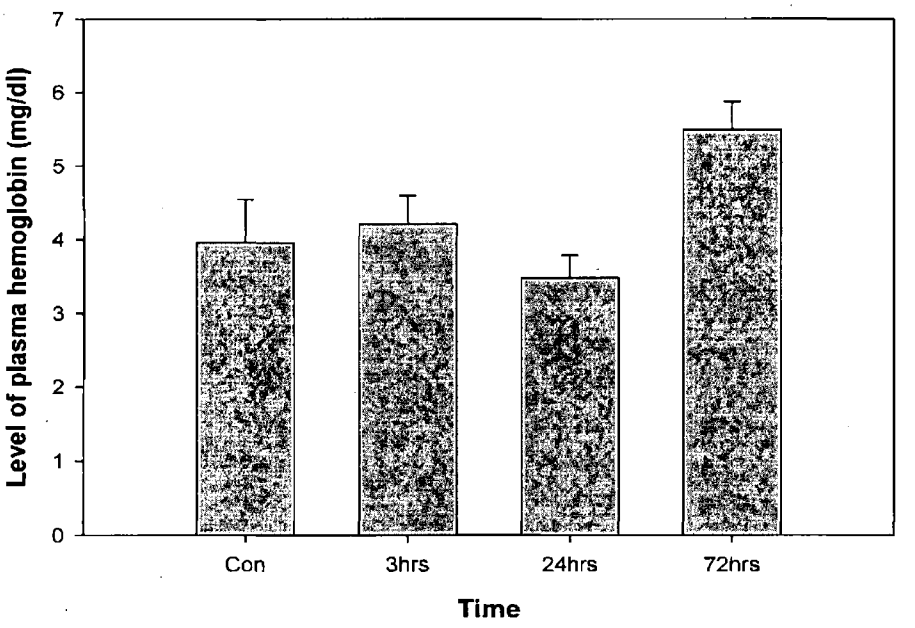

Fig.2. Plasma hemoglobin levels 3, 24, and 72 hours after SAH compared to control. Data presented as mean+/-SEM. Values are: control: 3.9 +/- 0.6, 3 hrs: 4.2+/- 0.4, 24 hrs: 3.5+/- 0.3, 72 hrs: 5.5+/- 0.4.

3.2. Intravital Microscopy

3.2.1. Physiology:

Subarachnoid hemorrhage in mice produced a sharp and immediate rise in intracranial pressure (ICP) to 91 +/- 29 mmHg (mean +/- STD). The mean arterial blood pressure (MABP) was lower in the mice with subarachnoid hemorrhage compared to sham operated mice, but still remained within the physiological threshold of cerebral autoregulation for the vast majority of the duration of intravital imaging (**table 1**). End tidal PCO2 values remained within close range in both SAH and sham groups throughout the experiments, and this resulted in arterial PCO2 which is within normal physiological range in both groups (**tables 2& 3**).

Table (1): Mean arterial blood pressure (MABP) values (mmHg) in sham and SAH mice (Mean +/- SEM) during IVM. Time (min) calculated from the start of IVM.

Time into IVM -->	15 min	30 min	45 min	60 min	75 min	90 min	105 min	120 min
Sham	78 +/- 5.7	79 +/- 8.2	80 +/- 6.7	77 +/- 7.5	79 +/- 7	76. +/- 8.6	82 +/- 7.9	79 +/- 10.6
SAH	68 +/- 5.4	69 +/- 3.9	63 +/- 4.3	64 +/- 2.5	71 +/- 7.1	57 +/- 2.4	60 +/- 2.7	54 +/- 2.7

Table (2): End tidal PCO2 values (mmHg) in sham and SAH mice (Mean +/- SEM) during IVM. Time (min) calculated from the start of IVM.

Time into IVM -->	15 min	30 min	45 min	60 min	75 min	90 min	105 min	120 min
Sham	26 +/- 0.4	27 +/- 0.7	28 +/- 0.6	27 +/- 0.5	27 +/- 0.7	26 +/- 0.7	26 +/- 0.9	26 +/- 0.8
SAH	26 +/- 1	26 +/- 0.9	27 +/- 0.7	27 +/- 1	27 +/- 0.9	26 +/- 0.8	26 +/- 0.9	26 +/- 0.8

Table (3): Arterial PCO2 (mmHg) in sham and SAH mice (Mean +/- SEM).

	Arterial PCO2
Sham	39 +/- 2.7
SAH	41 +/- 1.8

3.2.2. Distribution of blood

We were able to demonstrate the fact that the blood spreads from the source of bleeding at the circle of Willis at the base of the skull to the upper surface of cerebral cortex. Blood is seen as a dark layer encasing the left middle cerebral artery as it curls from the skull base to the upper cortical surface, and continues to spread along the branches of the middle cerebral artery in the subarachnoid space (**Fig. 2**). 75% of all vessel segments investigated showed a similar pattern. No blood was seen along the middle cerebral artery or any of its branches in the sham operated mice (**Fig. 3**).

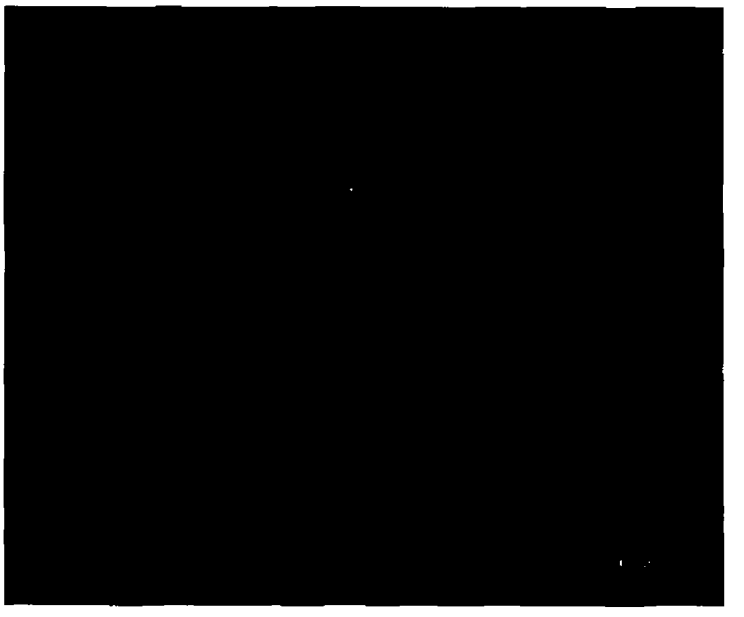

Fig. 2: Left middle cerebral artery and its two main branches after subarachnoid hemorrhage. Note: the blood is seen as the dark layer coating the MCA and its branches (red arrows).

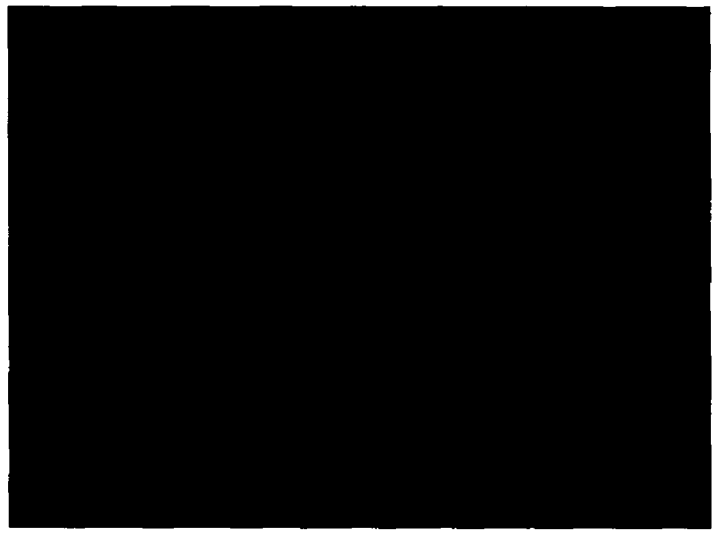

Fig. 3: Left middle cerebral artery and its two main branches after sham surgery. Note: there is no blood surrounding the MCA or any of its branches

3.2.3. Vasospasm

In the time window of 1-3 hours after subarachnoid hemorrhage, numerous vasoconstrictions were observed in various vessel segments. In total, 96 areas of vasoconstrictions were observed in 123 vessel segments. The vasoconstriction showed a very characteristic pattern, in the form of repeated areas of vasoconstriction interchanging with areas of normal vessel caliber in between (**Fig. 4, 5, 6 and 11**). Analysis of the severity of vasoconstriction, i.e. the degree to which the vascular diameter is diminished, showed 38.6% of all the vasospasms reduced the constricted vessel diameter to 80-90% of the normal diameter, 32% reduced the vascular diameter to 70-80% of its norm, 20.4% diminished the vas-

cular caliber to 60-70%, and 8% of the vasospasms causes reduction of the vascular caliber to 50-60% of normal caliber (**Fig. 7**).

In order to investigate the involvement of perivascular blood in the causation of vasospasm we analyzed vasospasms in the context of the presence or absence of perivascular blood. The vast majority of vasospasms occurred in the presence of perivascular blood; 93% of all vasospasm are associated with blood in the perivascular space compared to only 7% of vasospasm occurring in the absence of perivascular blood (**Fig. 8**).

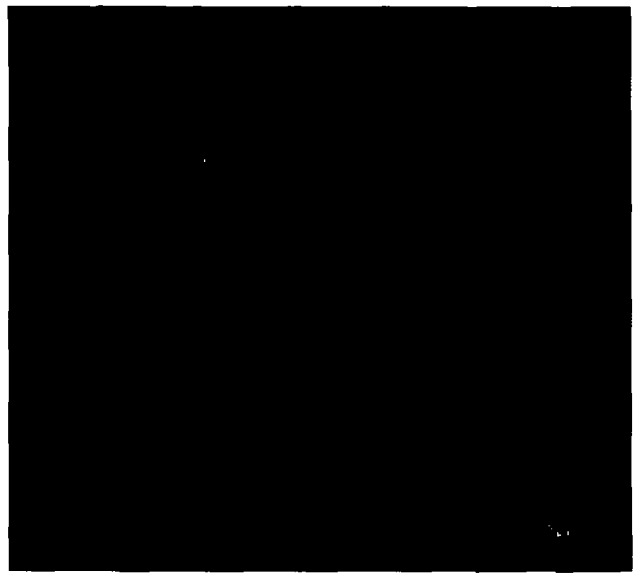

Fig. 4: Multiple vasospasms affecting an arteriole. Note the vasospasms (straight arrows) affect segments and spare the intervening segments. Presence of perivascular blood is also noted (twisted arrow).

Fig. 5: Multiple vasospasms: The pattern of vasospasm affecting segments and sparing the adjacent segments (yellow arrows). Note perivascular blood along the arteriole (Red arrows) affected by the spasms. Also note perivascular blood following the arteriole deep into the brain tissue.

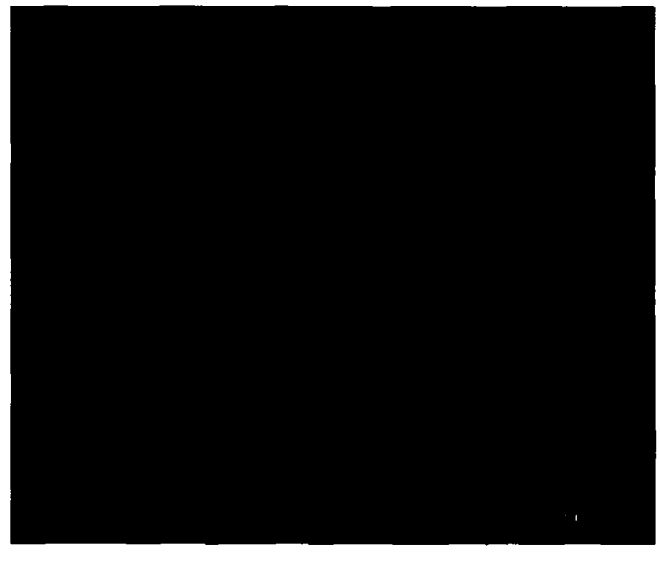

Fig. 6: Micovasospasm in an arteriole. Vasospasm affects a short segment (long arrow) of the arterioles, and sparing the adjacent segment. Note the presence of perivascular blood (short arrow).

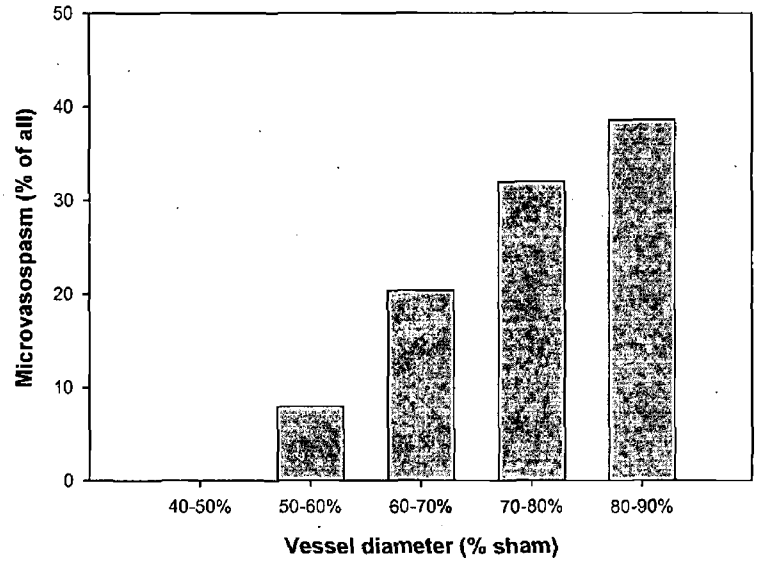

Fig. 7: The degree of vasospasm. Figures represent the percentage of narrowing at the constricted segments; i.e. the percentages of the vascular diameter at the constricted segment from the original vessel diameter. (n= 96 VS in 73 vessels).

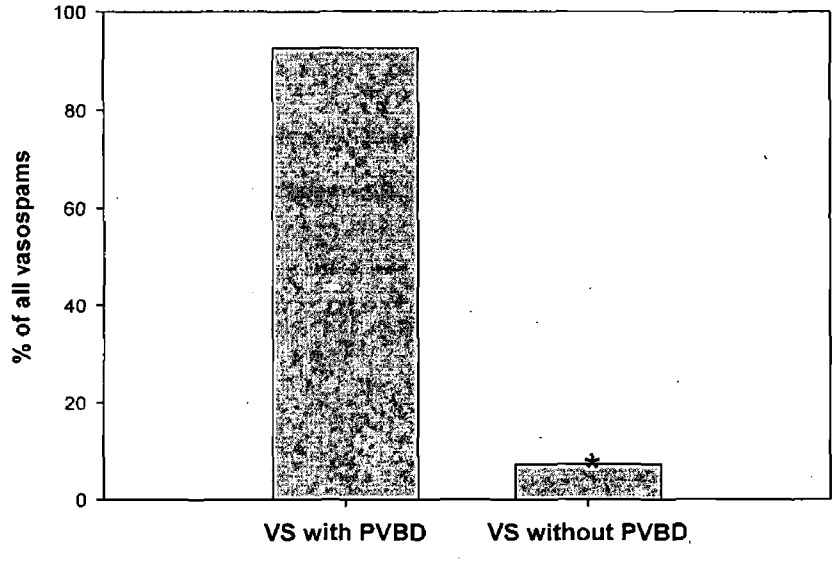

Fig. 8: Vasospasm (VS) in the presence of perivascular blood (PVBD) Vs vasospasm in the absence of PVBD. *P = 0.002 VS with PVBD (n= 96 VS in 73 vessels).

3.2.4. Microthrombi

Microthrombi were identified by accumulation of FITC-dextran in blood vessels, and were associated with cessation of blood flow in the respective blood vessel seen during imaging. They were seen as a lump of accumulated FITC-dextran (**Fig. 9**), or in the form of accumulated FITC dextran extending through the entire length of the microarterioles or capillaries (Fig. 10, 11 &12). A total of 34 microthrombi were detected during the period of imaging. Similar to vasospasm, a close correlation between the distribution of microthrombi and the presence of perivascular blood is noted. 79% of all micro thrombi were observed in vessels coated with perivascular blood, compared to 21% of micro thrombi detected in the absence of perivascular blood (**Fig. 13**).

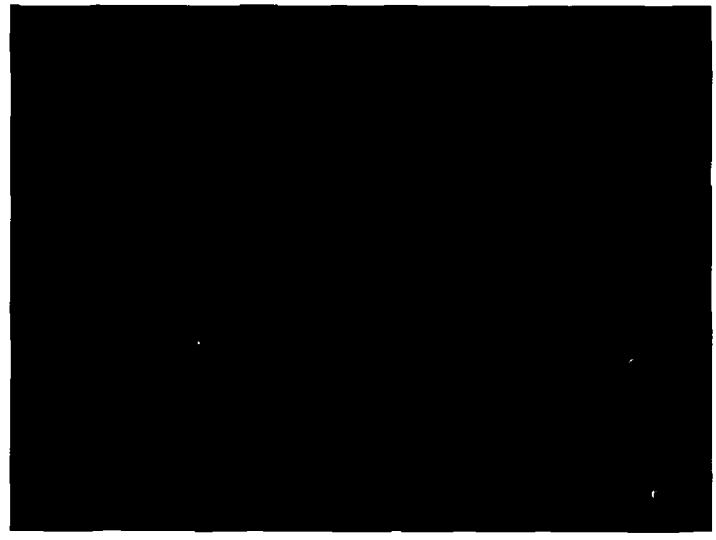

Fig. 9: Microthrombus in an arteriole: seen as focal accumulation of FITC dextran (red arrow).

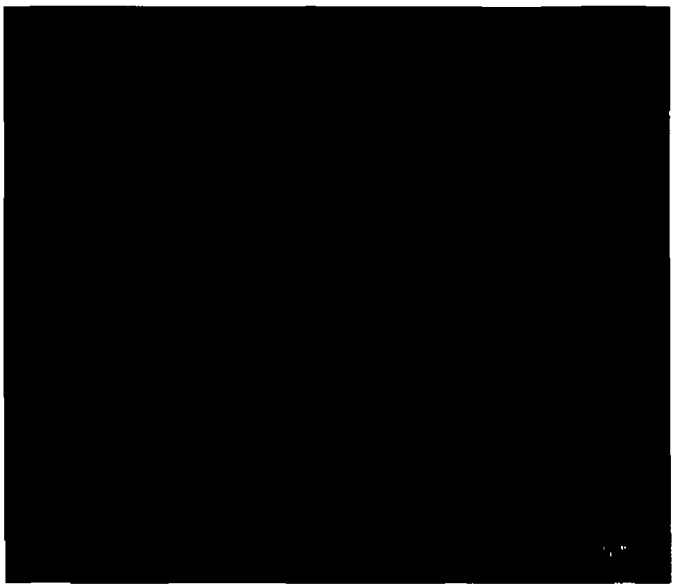

Fig. 10: Microthrombi: extensive microthrombi occupying the entire length of the capillaries (red arrows).

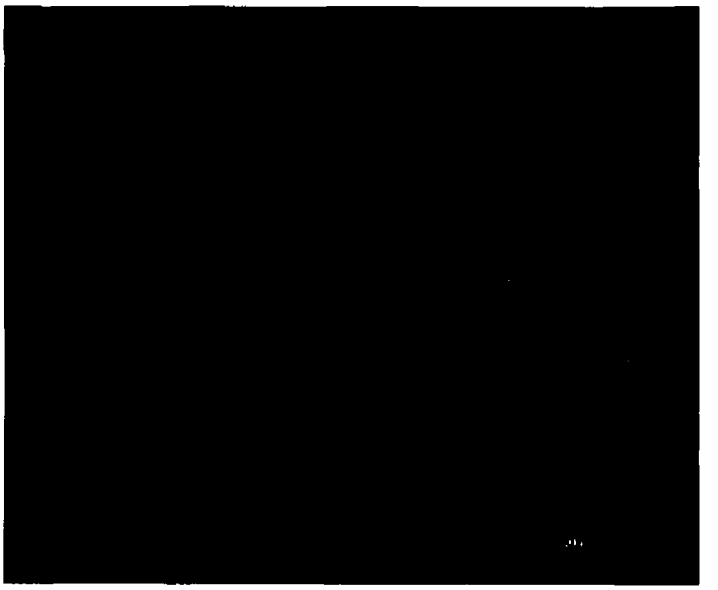

Fig. 11: Microthrombi completely occlude the entire arteriole/s (short arrows). Microvasospasms (long arrows (Right)) affecting two segments of the arteriole with normal segments in between. Note the presence of perivascular blood as dark layer adjacent to the arterioles (twisted arrows).

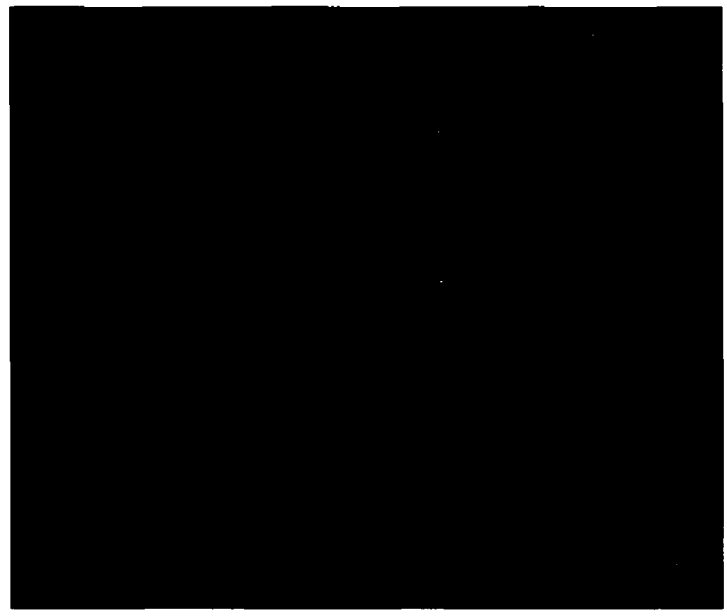

Fig. 12: Microthrombi (arrows) occluding the arteriole and extends into its branches.

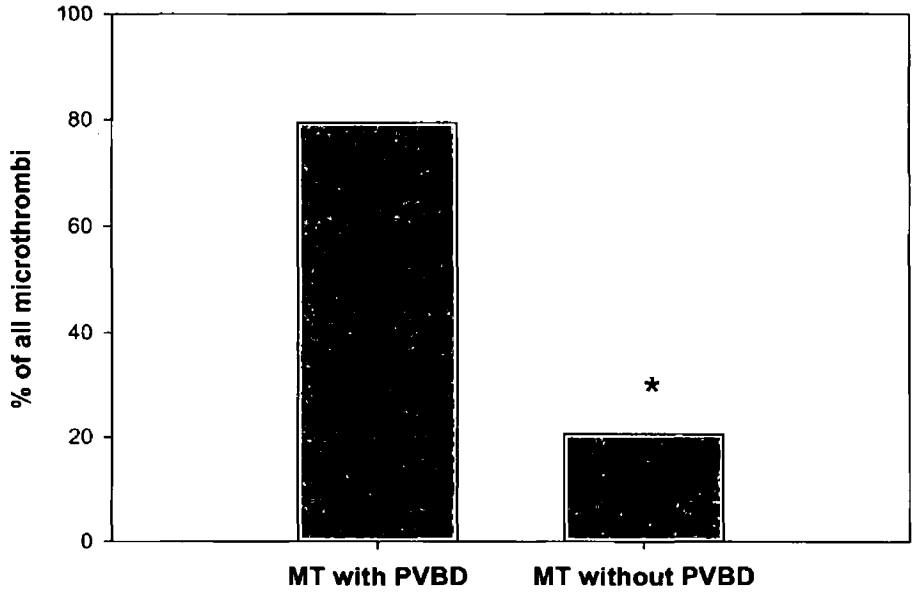

Fig. 13: Microthrombi (MT) in the presence of perivascular blood (PVBD) versus microthrombi in the absence of PVBD. * P= 0.026 Vs MT with PVBD. (n= 34 MT in 73 vessels).

3.2.5. Distribution of vasospasm and thrombosis according to vessel size

In order to determine the vessel category mostly affected by vasospasm and thrombosis, we analyzed the distribution of vasospasm and thrombosis among different vessel sizes. Our results showed that the vast majority of vasospasms and thrombi occurred in small arterioles and capillaries (**Fig. 14**). About 39% of all vasospasms were detected in vessels with 10-20 µm caliber, 28% of vasospasms occurred in vessels with 20-30 µm caliber and 10% in blood vessel size of 30-40 µm. Only 2% of vasospasms were seen in larger vessels (>60 µm in diameter). With regards to thrombi, 59% of them were seen in vessels <10 µm in diameter and 37% affected vessels with 10-20 µm in diameter. There were no thrombi seen any vessel larger than 30 µm in diameter (**Fig. 15**).

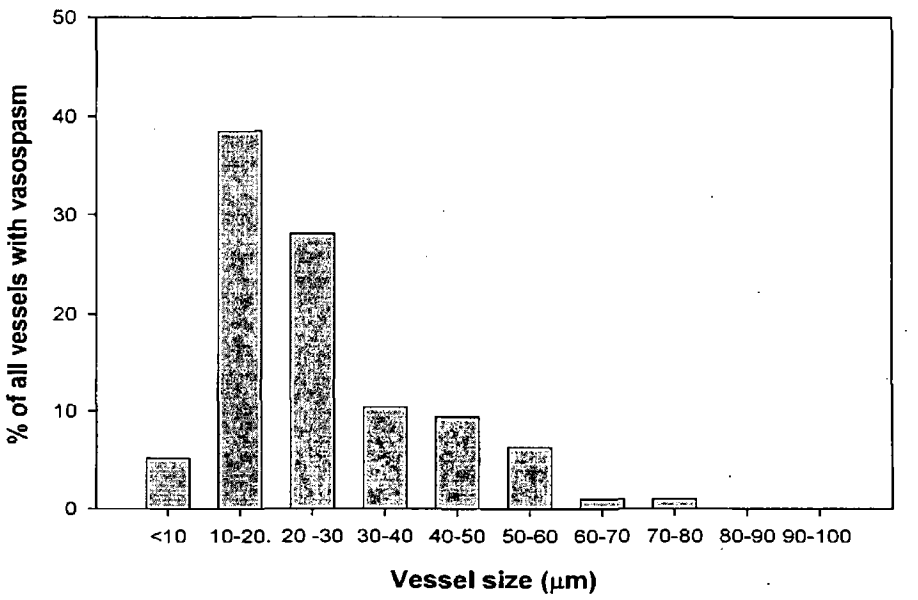

Fig. 14: Distribution of vasospasm among different vessel sizes. Vasospasm was predominantly detected in small arterioles and capillaries (diameter 10-30 µm). (n= 96 VS in 73 vessels)

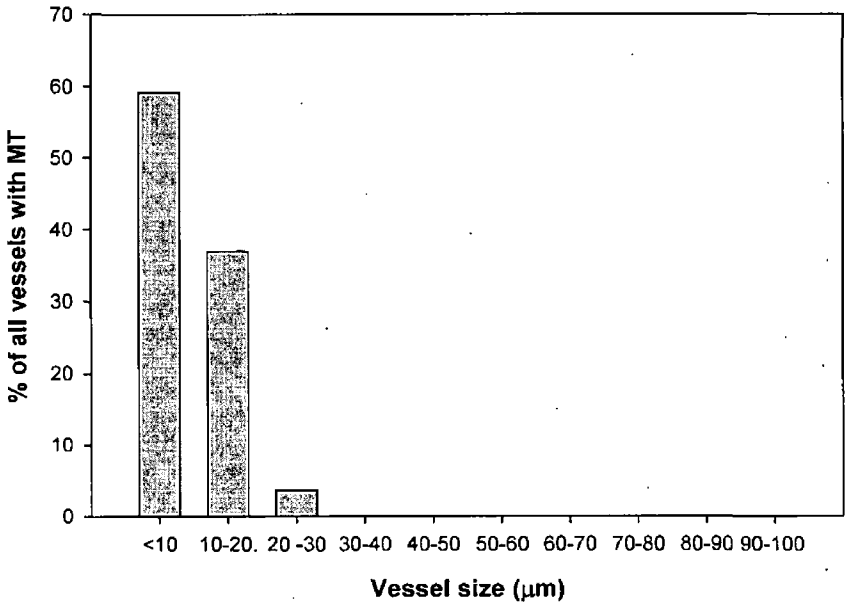

Fig. 15: Distribution of microthrombi (MT) among different vessel sizes. Thrombosis predominantly affects capillaries and small arterioles. (n= 34 MT in 73 vessels).

3.2.6. Perivascular blood and vasospasm and thrombosis

We also analyzed the association between the presence of perivascular blood and the development of vascular changes in the form of microvasospasms and/or microthrombi collectively. In this study only 34% of vessels segments which showed perivascular blood did not develop vasospasm or microthrombi. This means that though perivascular blood is not always associated with vascular changes, in two third of the cases its presence did coincide with the development of these vascular changes.

Since 75% of all segment investigated showed perivascular blood, we calculated the total percentage of segments with either microvasospasm or microthrombosis from all the segments investigated. We found 56% of all segments investigated showed either microvasospasm or microthrombosis.

Chapter 4

DISCUSSION

4.1. Plasma hemoglobin

There is little doubt that hemoglobin is released from slow hemolysis of the RBCs within the subarachnoid clot and is directly or indirectly responsible for the development of cerebral vasospasm after subarachnoid hemorrhage. NO depletion, which results from the scavenging effect of hemoglobin on NO (Goretski and Hollocher 1988; Ignarro 1990) and decreased production of NO through inhibition of NO synthesis (Nozaki, Moskowitz et al. 1993; Pluta, Thompson et al. 1996; Jung, Oldfield et al. 2007), is widely accepted as a potential mechanism by which hemoglobin causes post hemorrhagic cerebral vasospasm (Pluta ; Pluta, Dejam et al. 2005; Jung, Oldfield et al. 2007; Sehba and Bederson 2011). Identification of the levels of free hemoglobin within different body fluid compartments after subarachnoid hemorrhage augments the understanding of the mechanisms by which free hemoglobin can cause NO depletion, and subsequently lead to post hemorrhagic cerebral vasospasm. So far, hemoglobin and its metabolites have been detected in the subarachnoid clot (Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al.), the perivascular fluid (Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al.) and the cerebrospinal fluid (Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al. ; Barrows, Hunter et al. 1955; Suzuki, Muramatsu et al. 2003) in patients with aneurismal subarachnoid hemorrhage. Oxyhemoglobin has been found in the cerebrospinal fluid (CSF) as early as 2 hours after aneurismal subarachnoid hemorrhage, and continued to be detected for up to two weeks (Barrows, Hunter et al. 1955; Macdonald and Weir 1991). However, no correlation has been found between CSF oxyhemoglobin levels

and the severity of vasospasm (Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al.). The lack of correlation between CSF oxyhemoglobin concentration and the severity of cerebral vasospasm can be explained by the fact that lumbar CSF concentrations of oxyhemoglobin do not accurately reflect concentrations in the perivascular space immediately contiguous to spastic vessels. The results of the clinical studies measuring the perivascular hemoglobin concentrations after subarachnoid hemorrhage have been largely inconclusive (Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al.). Theoretically, the only other fluid in direct contact with the vascular wall, and could carry excess hemoglobin that could have an impact on the vascular physiology, is the plasma. Therefore, in this study, we measured the level of free hemoglobin in the plasma at three different time points during the first 72 hours after subarachnoid hemorrhage. Our data showed the presence of normal plasma free hemoglobin concentrations for up to 72 hour after subarachnoid hemorrhage. This is an important observation which can lead to valuable clues in the implication of free hemoglobin in the etiology of acute post- hemorrhagic cerebral vasospasm. The presence of normal intravascular hemoglobin concentration after subarachnoid hemorrhage, which shown in our data, excludes direct inhibition of endothelial NOS by plasma hemoglobin as a possible mechanism involved in NO depletion. This conclusion prompted us to investigate the role of perivascular hemoglobin that is present in the perivascular hematoma as a major player in NO depletion from the vessel wall. Our findings and conclusions in this regard are part of the findings of the intravital microscopy study, which is described below.

4.2. Intravital microscopy

The main purpose of conducting the intravital microscopy study was to closely examine the cerebral microcirculation for the presence of microvascular ischemic changes in the immediate aftermath of experimental subarachnoid hemorrhage. Our findings confirmed the presence of pearl-and -string like vasoconstrictions affecting almost half of all the vessel segments investigated within the interval of 1-3 hours after SAH. At these constrictions, the vascular diameter was reduced to an extent which could lead to reduced blood flow. Another significant observation from this study is the presence of microthrombi within microarterioles and capillaries at such an early time point after SAH. The presence of microthrombi as early as 10 minutes after subarachnoid hemorrhage has been described in histological sections after experimental subarachnoid hemorrhage in mice (Sehba, Mostafa et al. 2005). In this study, we were able to demonstrate microthrombosis along with microvasospasm under physiological conditions in living mice, thus avoiding all the shortcomings of other none in vivo models.

The main strength of this study is that two well established methods were used to conduct the study. For induction of experimental subarachnoid hemorrhage we used the endovascular perforation model, which has the advantage of resembling human aneurismal subarachnoid hemorrhage in having an injured artery leading to hemorrhage under direct arterial blood pressure. For imaging of cerebral vasculature after subarachnoid hemorrhage, the intravital fluorescence microscopy technique was used. The combined use of these two techniques, as

described, has enabled us to study cerebral microcirculation under controlled physiological conditions. The physiological parameters that could directly affect cerebral vasculature were continuously monitored and kept within their normal range throughout each experiment. The mean arterial blood pressure (MAP) was constantly at levels within the cerebral autoregulation threshold in both experimental groups, and its values were within similar range in both the sham and SAH groups (**Table 1**). End tidal PCO2 levels were controlled by adjusting the ventilation during the experiments and served as an index for arterial PCOs levels. Thus, the levels of arterial PCO2 measured at the end of each experiment were within the normal physiological range in both experimental groups (**Tables 2 & 3**). Therefore, we are confident that all the necessary precautions were taken to avoid any influence of the current experimental methods on the pathophysiological processes under investigation.

4.3. Microvasospasm and microthrombosis

The findings of this study demonstrate the presence of ischemic changes in the form of vasoconstriction and thrombosis at the microcirculatory level as early as 1 hour after experimental subarachnoid hemorrhage. These findings support the hypothesis suggested by Bederson (Bederson, Germano et al. 1995; Bederson, Levy et al. 1998) and others (Schubert, Seiz et al. 2011) that cerebral ischemia after subarachnoid hemorrhage is caused by vasoconstriction at the level of cerebral microcirculation. There have been few recent clinical studies reaching

the same conclusion i.e. vasoconstriction, at the level of microcirculation, plays an important role in the causation of post hemorrhagic cerebral ischemia (Schubert, Seiz et al. 2009; Schubert, Seiz et al. 2011). Moreover, Uhl et al, actually, demonstrated microvasospasms affecting cerebral microcirculation in patients with aneurismal subarachnoid hemorrhage (Uhl, Lehmberg et al. 2003). However, none of these clinical studies were done within the very first few hours following the hemorrhage, and hence, little is known about the pathological events that occur immediately after aneurismal subarachnoid hemorrhage in humans. Revisiting the statistics of the outcome of aneurismal SAH shows that 16% of all patients who suffers from SAH die before they reach medical care, which practically implies they die within the first few hours following SAH (Pobereskin 2001; Macpherson, Lewsey et al. 2011). This fact shows the prognostic importance of the pathological events within this short period of time after SAH and the need to understand these events in order to develop appropriate therapeutic measures that will help improve the outcome of these patients. Our data have shown that within an hour after experimental subarachnoid hemorrhage, large areas of the middle cerebral artery microcirculation are affected by microvasospasm and microthrombosis severe enough to cause cerebral ischemia. This conclusion is drawn from two main observations noted from our data. First, a closer look at the percentage of reduction of the vascular diameter at the sites of vasospasms shows that 28.4% of all vasospasms resulted in at least 30% reduction of the vascular diameter, and a further 32% of all vasospasms has led to a reduction of the vascular diameter by 20-30% (**Fig. 7**).The effects of

such reductions in vascular diameter on blood flow within any particular blood vessel can be drawn from Hagen- Poiseuille law, which indicates that vascular blood flow decreases by r^4 (r is the diameter). Hence, reduction of the vascular diameter by, for example, 30% will reduce vascular blood flow by 81% indicating marked reduction of flow in blood vessels affected by such degree of vasospasms. The effect of reduction of vascular diameter on blood flow is expected to be exacerbated by microthrombi, the presence of which indicates occlusion of the vascular lumen and cession of blood flow within the affected segments irrespective of the vascular diameter. Overall, 56% of all the vessel segments investigated in this study were affected by either vasospasm or thrombosis (or both). Second, both vasospasm and thrombosis predominantly affected the small arterioles and capillaries (**Fig.14 & 15**), and because these are mostly end arteries, their occlusion is likely to result in cerebral ischemia. Whether the same vascular changes happen during the same period of time in patients with aneurismal subarachnoid hemorrhage and to the same extent and severity remains to be seen.

Microthrombosis has also been reported in a number of clinical studies in patients with aneurismal subarachnoid hemorrhage. In a very interesting autopsy study published in 2006 on 29 patients who died from aneurismal subarachnoid hemorrhage, Stein et al. demonstrated that microthrombi are present in abundance in these patients, and their presence is strongly correlated with clinical and pathological signs of ischemia (Stein, Browne et al. 2006). These autopsy findings were further supported by radiological studies in living patients with an-

eurismal SAH showing widespread radiological changes across both cerebral hemispheres consistent with microembolic occlusion of cerebral microcirculation (Kivisaari, Salonen et al. 2001; Romano, Forteza et al. 2002; Rabinstein, Weigand et al. 2005). The strongest evidence was reported by Romano et al., who demonstrated microembolic signals on transcranial Doppler ultrasonography studies in 16 out of 23 patients (70%) with aneurismal subarachnoid hemorrhage (Romano, Forteza et al. 2002). These autopsy and radiological evidence of widespread microthrombosis in cerebral microcirculation in patients with aneurismal subarachnoid hemorrhage are supported by several hematological studies which showed activation of coagulation system as well as fibrinolytic system early during the course of aneurismal subarachnoid hemorrhage (Fujii, Takeuchi et al. 1995; Peltonen, Juvela et al. 1997; Nina, Schisano et al. 2001; Morga, Czepko et al. 2007). Morga et al. measured the plasma thrombin-antithrombin-3 complex (TAT) (a marker for coagulation system), and D- dimmers (markers for fibrinolytic system) in 72 patients with aneurismal SAH at the time of admission, and 7 days after aneurysm surgery, and compared them with to those of 84 patients without SAH. They concluded that SAH causes activation of both coagulation and fibrinolytic system and such activation is associated with poor clinical status at the time of admission and poor outcome later (Morga, Czepko et al. 2007). Similar findings were reported by Nina et al., who showed evidence of early activation of coagulation and fibrinolytic system in a series of 76 patients with SAH, and found a strong correlation between the plasma thrombin-antithrombin-3 (TAT) and D dimmers levels and the clinical outcome of

these patients (Nina, Schisano et al. 2001). Few other studies have confirmed this strong correlation between the scale of derangement of hemostatic cascades and the clinical outcome of patients with aneurismal subarachnoid hemorrhage (Ilveskero S Fau - Juvela, Juvela S Fau - Siironen et al. ; Kusanagi H Fau - Teramoto, Teramoto A Fau - Shimura et al. ; Itoyama, Fujioka et al. 1994; Fujii, Takeuchi et al. 1995). Such is the extent of this correlation that some authors regard markers of coagulation state in the early periods after aneurismal subarachnoid hemorrhage as independent factors in determining the patients' outcome after aneurismal subarachnoid hemorrhage (Fujita K Fau - Tamaki and Tamaki ; Juvela S Fau - Siironen and Siironen ; Nina, Schisano et al. 2001; Morga, Czepko et al. 2007). (Sehba, Mostafa et al. 2005). Studies that compare the levels of these markers in the CSF and jugular venous blood to that of systemic venous blood have shown that these markers are much higher in the CSF and jugular venous blood compared to systemic levels, indicating that the hypercoagulability state after subarachnoid hemorrhage originates from the cerebral circulation (Denton, Robertson et al. 1971; Juvela, Hillbom et al. 1991). The mechanisms responsible for activation of coagulation system after aneurismal subarachnoid hemorrhage are not clear. It's speculated to be caused by release of tissue thromboplastins from damaged brain tissue into the circulation (Yamamoto, Clower et al. 1991; Fujii, Takeuchi et al. 1995). Platelets function in humans during the early stages after aneurismal subarachnoid hemorrhage is poorly understood. Most of the data in literature on platelets function in patients with aneurismal subarachnoid hemorrhage came from Juvela and colleagues in

Helsinki, Finland. They have shown, in numerous studies, increased platelets aggregation and thromboxane release during the course of delayed cerebral vasospasm, suggesting a causative role for platelets aggregation and thromboxane in the development of delayed cerebral vasospasm and delayed ischemic neurological deficit in patients with aneurismal subarachnoid hemorrhage (Juvela 1990; Juvela, Kaste et al. 1990; Juvela, Kaste et al. 1990; Juvela, Ohman et al. 1991). There are similar reports of platelets activation and aggregation at later stages after experimental subarachnoid hemorrhage in animals (Denton, Robertson et al. 1971; Ohkuma, Suzuki et al. 1990; Ohkuma, Ogane et al. 1993; Pisapia, Xu et al. 2011). However, Platelets aggregates in the lumen of small parenchymal vessels causing perfusion deficit have been demonstrated as early as 10 minutes after experimental subarachnoid hemorrhage in two studies by Sehba et al. (Sehba, Mostafa et al. 2005; Friedrich, Flores et al. 2010). Interestingly, they reported the presence of constricted vessel segments in otherwise normal vessels located next to these platelets aggregates (Friedrich, Flores et al. 2010). The mechanisms behind platelets activation and aggregation following subarachnoid hemorrhage are not fully understood. Endothelial injury, leading to loss of normal endothelial inhibitory effect on platelet aggregation, is regarded as a potential mechanism responsible for platelets aggregation in parenchymal vessels after SAH (Ohkuma, Suzuki et al. 1990; Ohkuma, Ogane et al. 1993; Akopov, Sercombe et al. 1996). Another important mechanism thought to play a key role in platelets activation and aggregation after subarachnoid hemorrhage is NO depletion (Sehba, Schwartz et al. 2000; Friedrich, Flores et al. 2010). Un-

der normal physiological conditions, NO inhibits platelets adhesion and aggregation. Therefore, depletion of NO from the vessel wall, which is known to occur after SAH, will likely remove its inhibitory effects on platelets adhesion and aggregation, leading to platelets aggregation within the vascular lumen (Friedrich, Flores et al. 2010).

Two previous experimental studies (Friedrich, Flores et al. 2010; Friedrich, Muller et al. 2011) have shown that, microthrombi and microvasospasms are predominantly found adjacent to each other in the same vessel. This observation suggests either a common pathology is mediating both microvasospasm and microthrombosis, or they both play a part in the formation of one another. It's also possible that both scenarios occur at the same time. NO depletion is likely to be the common pathology that has the potential to result in the formation of both. Oxyhemoglobin induced NO depletion from the vascular wall is now seen as the most likely mediator of post hemorrhagic microvasospasms (Jung Cs Fau - Iuliano, Iuliano Ba Fau - Harvey-White et al. ; Pluta ; Nozaki, Moskowitz et al. 1993; Pluta, Dejam et al. 2005; Jung, Oldfield et al. 2007; Sehba and Bederson 2011). It's also believed that lack of NO increases platelets activation and aggregation (Yamamoto, Clower et al. 1991; Friedrich, Flores et al. 2010), and alters the antithrombotic properties of the endothelial cells (Yamamoto, Clower et al. 1991; Ohkuma, Ogane et al. 1993); both actions can lead to the formation of microthrombi in the vascular lumen. Reduction of the vessel diameter at the site of microvasospasm leads to reduced blood flow through the vessel lumen, and if that is severe enough, it can cause stasis of blood proximal to the constriction.

As we know, stasis is a strong predisposing factor for the formation of thrombi within any given blood vessel. Once a microthrombus is formed it can either remain at the same size, or increase in size and occupy a longer segment of the arteriole through the phenomena of thrombus propagation. This explains the two types of microthrombi we encountered in this study (**Fig. 9-12**). The observation that microthrombi were detected mainly at the proximal end of the constricted segments perfectly support this hypothesis. On the other hand, microthrombi can also cause or potentiate the formation of microvasospasm (Okada, Copeland et al. 1994; Park, Yamaguchi et al. 2004). Platelets activation and aggregation which occur at the site of microthrombi lead to release of thromboxanes (Juvela 1990; Juvela, Kaste et al. 1990; Juvela, Kaste et al. 1990; Juvela, Ohman et al. 1991) and other potent vasoconstrictors such as ADP, serotonin and PDGF (Okada, Copeland et al. 1994; Park, Yamaguchi et al. 2004) from platelets into the surrounding, and these are carried distal to the thrombus, where the vasoconstrictions occur. The most likely scenario is that oxyhemoglobin induced NO depletion causes both microvasospasm and vasoconstriction, and both then act in a synergistic manner to potentiate each other. This hypothesis is supported by our findings and the findings of others, which showed that there is a strong correlation between the distribution of both microvasospasms and microthrombi (Honma, Clower et al. 1989; Yamamoto, Clower et al. 1991; Stein, Browne et al. 2006) and the presence of perivascular or subarachnoid blood, and also by the fact that the status of coagulation after aneurismal subarachnoid hemorrhage correlates well with the amount of sub-

arachnoid blood as shown in many studies (Fujii, Takeuchi et al. 1995; Morga, Czepko et al. 2007).

4.4. Perivascular blood and the etiology microvasospasm and microthrombosis

The second aim of the intra-vital microscopy study was to investigate the role of perivascular hemoglobin in the etiology of NO depletion that will eventually results in microvasospasm and microthrombosis, by estimating the degree of correlation between the distribution of mirovasospasms and microthrombi and the presence of perivascular blood. Our findings showed a very strong correlation between the presence of microvasospasm and microthrombi in the blood vessels and the presence of perivascular blood adjacent to the vessel wall. The contents of perivascular blood were examined by Pluta and colleagues in a model of subarachnoid hemorrhage where they place clotted arterial blood over the right middle cerebral artery in monkeys. They found the levels of oxyhemoglobin and deoxyhemoglobin in the perivascular fluid increase during the development of delayed cerebral vasospasm (Pluta, Afshar et al. 1998). Despite the limitations of the model used in comparison with the endovascular perforation model, and their focus on delayed cerebral vasospasm, the results of their study provide a strong link between elevated level of perivascular hemoglobin concentration and cerebral vasospasm. Our study is the first to describe a closer correlation between acute microcirculatory ischemic changes and the presence of perivascular blood in direct contact with the adventitia of blood ves-

sels. By establishing this strong link between perivascular blood and the development of microvasospasm and microthrombi, and taking into account the presence of normal plasma hemoglobin levels at the time of these observations, we add to the evidence supporting the theory that, blood and blood products, present in the perivascular space and coating cerebral arterioles, play a causative role in the etiology of microvasospasm, and microthrombi, leading to occlusion of cerebral microcirculation, which is ultimately responsible for post hemorrhagic cerebral ischemia in patients with SAH. These findings are consistent with those of Fisher and Kistler et al. who have shown, in two consecutive studies, that the volume and distribution of blood within the subarachnoid space determine the distribution and the severity of vasospasm in patients with SAH (Fisher, Kistler et al. 1980; Kistler, Crowell et al. 1983; Findlay, Macdonald et al. 1991). They concluded from their studies that, by quantifying the subarachnoid blood volume on the CT scan of these patients we can accurately predict whether or not they are going to develop vasospasm and, accordingly, ensure appropriate prophylactic measures against cerebral vasospasm. In their famous autopsy study; Stein et al. found a strong correlation between the presence of microthrombi and the overlying subarachnoid blood (Stein, Browne et al. 2006). The extent of activation of the coagulation and fibrinolytic cascade, which is seen early after aneurismal subarachnoid hemorrhage is also shown to be proportionate to the amount of subarachnoid blood identified on the CT scans of these patients (Fujii, Takeuchi et al. 1995; Morga, Czepko et al. 2007). There is further evidence in the literature which can explain the mechanisms by which perivascular hemo-

globin can cause NO depletion. Intense NOS activity has been detected in the nerve endings in the adventitia of cerebral vessels of monkeys (Pluta, Thompson et al. 1996), rats and humans (Nozaki, Moskowitz et al. 1993), adding another important source of NO for the vessel wall. These adventitial NOS containing nerves mediates neurogenic cerebral vasodilatation (Toda and Okamura 1990; Toda and Okamura 1991; Nozaki, Moskowitz et al. 1993; Pluta, Thompson et al. 1996). It has been shown that these nerve endings are damaged in vessels exposed to blood or oxyhemoglobin (Toda and Okamura 1990). Furthermore, more recent reports confirmed the disappearance of NOS activity from the adventitial nerve endings in cerebral vessels in spasm, while at the same time immunoreactivity of endothelial NOS was preserved in these vessels (Pluta, Thompson et al. 1996) . This will clearly suggest the loss of vasodilatory input from the adventitia in response to the presence of blood in the perivascular space, is a key element in the etiology of post hemorrhagic cerebral vasospasm. These findings, together with the findings of Fisher and Kistler et al. of a rather localized nature of post hemorrhagic cerebral vasospasm, mostly in areas with subarachnoid blood, are inconsistent with an exclusively intravascular origin for the etiology for vasospasm, as intravascular pathologies are more likely to be more generalized. The results of our two studies together, i.e. the strong correlation between the presence of perivascular blood and the development of microvasospasm and microthrombosis, and the observation of normal plasma hemoglobin level at the time of vasospasm and microthrombosis, are also inconsistent with an intravascular pathology. Hence, NO depletion after subarach-

noid hemorrhage is unlikely to be due inhibition of endothelial NO synthase by increased intravascular hemoglobin. Perivascular hemoglobin, scavenging the available NO and, at the same time, inhibiting NOS in the adventitial nerve endings leading to depletion of NO from the vessel wall, seems to be, in our opinion, the more likely mechanism of NO depletion, which is thought to be responsible for acute cerebral vasospasm after subarachnoid hemorrhage. The potential therapeutic implication of the extra-vascular pathogenesis of post hemorrhagic cerebral vasospasm is that any therapeutic agent has to be able to reach the perivascular space to have the desired effects.

4.5. Oxyhemoglobin: the principle agent responsible for vasospasm

The hemoglobin metabolites which have been linked with a possible role in cerebral vasospasm are oxyhemoglobin, bilirubin and methemoglobin. Methemoglobin is a product of autoxidation of oxyhemoglobin, while bilirubin is the result of enzymatic breakdown of oxyhemoglobin. Several studies have shown bilirubin does not possess vasospasmatic action on cerebral arteries in vitro (Macdonald and Weir 1991). Although elevated levels of bilirubin have been detected in CSF of patients with SAH, these levels were not associated with vasospasm (Suzuki, Muramatsu et al. 2003). Most in vivo studies on methemoglobin concluded that it has no vasoconstriction effect on cerebral arteries (Macdonald, Weir et al. 1991). Oxyhemoglobin, on the other hand, showed definite spasmodic action when applied to cerebral arteries (Macdonald,

Weir et al. 1991), and its levels were elevated in the perivascular fluid of cerebral arteries affected by vasospasm (Pluta, Afshar et al. 1998). Therefore, of all the hemoglobins, oxyhemoglobin is regarded as the principal pathogen responsible for alteration of vascular physiology after subarachnoid hemorrhage (Asano ; Kajikawa H Fau - Ohta, Ohta T Fau - Yoshikawa et al. ; Macdonald, Weir et al. 1991; Pluta, Oldfield et al. 1997). The mechanisms by which oxyhemoglobin causes vasospasm, are not fully understood. Possibilities include: scavenging (Goretski and Hollocher 1988; Ignarro 1990; Sehba, Schwartz et al. 2000) and decrease production (Martin, Villani et al. 1985; Sehba, Schwartz et al. 2000) of NO, the release of free radicals (Misra and Fridovich 1972; Gao, Ding et al. 2009; Munakata, Ohkuma et al. 2011), the initiation and propagation of lipid peroxidation causing increased production of vasoactive eicosanoids and endothelins (endothelin 1) from arterial wall (Toda 1990), induction of structural damage to the arterial wall (Ostrowski, Colohan et al. 2006), stimulation of neuronal apoptosis (Cahill, Calvert et al. 2006; Cheng, Wei et al. 2009), and evoking of an inflammatory reaction through increased expression of cell adhesion molecules which leads to rolling, adhesion and extravasation of leukocytes (Momin, Schwab et al. 2009). These actions are complex and closely connected and ultimately result in imbalance of the autoregulation of the vascular tone in favor of vasoconstriction.

4.6. Clinical trials against vasospasm

The mechanisms of action of oxyhemoglobin, which are proposed to explain its role in the etiology of vasospasm, have been the basis of clinical trials looking for effective therapies that can reduce the incidence of post hemorrhagic cerebral vasospasm and improve the outcome of patients with aneurismal subarachnoid hemorrhage. The most recent example is the endothelial receptor antagonist, clazosentan, clinical trials (Vajkoczy, Meyer et al. 2005; Macdonald, Kassell et al. 2008; Macdonald, Higashida et al. 2011). These trials are based on the observations that oxyhemoglobin causes increased production of endothelin -1, which is a potent vasoconstrictor protein (Kasuya, Weir et al. 1993), and the findings of elevated levels of endothelin-1 in the cerebrospinal fluid of human and animals with post hemorrhagic cerebral vasospasm (Pluta Rm Fau - Boock, Boock Rj Fau - Afshar et al. ; Seifert, Loffler et al. 1995; Zimmermann and Seifert 1998). The latest report on these trials is phase 3 of randomized double-blind, placebo- controlled trial of the endothelin-1receptor antagonist, clazosentan (Macdonald, Higashida et al. 2011). This is a large multi-center clinical trial involving 1157 patients with aneurismal subarachnoid hemorrhage, in 27 countries, over a period of 18 months. Despite significant reduction of the incidence of moderate to severe vasospasm in patients treated with clazosntan in this trial, there was no significant improvement in morbidity or mortality or functional outcome. This efficacy against angiographic vasospasm, which did not translate into improved clinical outcome, have prompted the investigators in this clinical trial to conclude that, factors other than delayed cerebral vasospasm

contribute to ischemic brain injury and poor functional outcome after aneurismal subarachnoid hemorrhage. These factors, in their opinion, are likely to be microcirculatory dysfunction and early brain injury. The findings of our current study point towards a similar conclusion; i.e. microcirculatory failure, due to vasospasm and thrombosis across cerebral microcirculation in the acute stages of subarachnoid hemorrhage, is a major contributor to ischemic brain injury and poor functional outcome after aneurismal subarachnoid hemorrhage.

Other clinical trials based on other mechanisms of action of oxyhemoglobin have also been unsuccessful in improving patients' outcome. Tirilazad, a free radical scavenger and an inhibitor of lipid peroxidation, was used in five randomized, double-blind and placebo-controlled clinical trials to assess its effects on unfavorable outcome, symptomatic vasospasm and cerebral infarction after aneurismal subarachnoid hemorrhage (Kassell, Haley et al. 1996; Haley, Kassell et al. 1997; Lanzino and Kassell 1999; Lanzino, Kassell et al. 1999). The results from these trials showed that although tirilazad did decrease the incidence of angiographic vasospasm, it did not improve the clinical outcome of patients with aneurismal subarachnoid hemorrhage (Jang, Ilodigwe et al. 2009). Statins, which have an anti-inflammatory effects and upregulate endothelial NO synthase, have also been used in clinical trials to test its efficacy against angiographic vasospasm and poor functional outcome after aneurismal subarachnoid hemorrhage (Lynch, Wang et al. 2005; Tseng, Czosnyka et al. 2005; Tseng, Hutchinson et al. 2007; Chou, Smith et al. 2008; Vergouwen, de Haan et al. 2010). The results from these trials were conflicting, but overall it did not have

beneficial effects on angiographic vasospasm or the patients' outcome (Vergouwen, de Haan et al. 2010).

4.7. Nitric oxide donors: promising therapeutic potential

The hypothesis that hemoglobin induced NO depletion is responsible for post hemorrhagic cerebral vasospasm has gain support from the success of therapies based on repletion of NO in tackling vasospasm and improving outcome of patients with aneurismal SAH. Administration of nitric oxide, through various routes, successfully ameliorates the development of vasospasm both in clinical and experimental settings (Thomas Je Fau - Rosenwasser and Rosenwasser ; Egemen, Turker et al. 1993; Thomas, Nemirovsky et al. 1997; Pluta, Dejam et al. 2005). Afshar et al. showed, in an experimental subarachnoid hemorrhage in monkeys, that intracarotid infusion of NO solution increases cerebral blood flow, decreases vascular resistance and reverse angiographic vasospasm without producing profound systemic hypotension (Afshar, Pluta et al. 1995). More recently, Momin et al. implanted an NO donor molecule (diethylenetriamine (DETA)/NO incorporated into ethylene/vinyl acetate (EVAc) polymers) into the cisterna magna of mice that are genetically susceptible to post hemorrhagic vasospasm (haptoglobin 2-2 mice), to evaluate the role of continuous delivery of NO on vasospasm. In this study, they shown that controlled release of NO into the subarachnoid space prevents vasospasm and, more importantly, minimizes neurological deficit in these mice (Momin, Schwab et al. 2009). These encourag-

ing results of NO donors from experimental studies were translated into clinical research, which is so far showing promising results.

Various preliminary clinical studies on small numbers of patients using intrathecal or intraventricular sodium nitroprusside, an NO donor molecule, for the treatment of severe post hemorrhagic vasospasm refractory to conventional therapy have, indeed, shown good to excellent outcome (Thomas and McGinnis 2002; Pathak, Mathuriya et al. 2003; Agrawal, Patir et al. 2009). Raabe et al. studied the effects of intraventricular administration of sodium nitroprusside on cerebral hemodynamics in 13 patients suffering from severe refractory vasospasm following aneurismal subarachnoid hemorrhage (Raabe, Zimmermann et al. 2002). In 6 of the 13 patients enrolled in the study, intraventricular sodium nitroprusside produced a marked improvement in cerebral circulation time (CCT) and brain tissue oxygenation (Pbro2), reflecting an improved cerebral blood flow (Raabe, Zimmermann et al. 2002). In another study, Thomas et al. studied the effects of intrathecal sodium nitroprusside (ITSN) on treatment and prevention of angiographic vasospasm and ischemic neurological deficit in 21 patients with poor grade aneurismal subarachnoid hemorrhage (Thomas, Rosenwasser et al. 1999). In 12 patients in this study, ITSN was administered after the onset of severe angiographic vasospasm and resulted in reversal of the vasospasm, decreased cerebral circulation time and improved clinical outcome. In 9 other patients ITSN was administered prophylactically and was successful in preventing vasospasm and ischemic neurological deficits. All the patients in this study have favorable outcome (Thomas, Rosenwasser et al. 1999). In a similar study

Agrwal et al. used intraventricular sodium nitroprusside to treat 10 patients with poor grade subarachnoid hemorrhage (Hunt and Hess grade 2 and higher) and compared the outcome to 10 patients with similar grades who served as controls (Agrawal, Patir et al. 2009). All 10 patients who received the IVSN showed improved cerebral blood flow on transcranial Doppler studies, and the overall clinical outcome was good or excellent in 7 of those 10 patients (Agrawal, Patir et al. 2009) . Although a substantial vasodilation of large-caliber cerebral vessels in response to SNP was demonstrated in these studies, its beneficial effects are more likely to be due to its vasodilatory effects on small blood vessels at the level of cerebral microcirculation. This is reflected by improvement of collateral circulation, shortening of circulation time, sometimes without dramatic change in the diameter of the large-caliber vessels, which have also been noted after administration of SNP in these studies (Thomas, Rosenwasser et al. 1999; Raabe, Zimmermann et al. 2002; Agrawal, Patir et al. 2009). The predominant effect of SNP on small blood vessel which is concluded from these studies is consistent with the nature of NO molecule as the smallest biologically active molecule in nature, making it capable of penetrating tissues directly (Thomas, Rosenwasser et al. 1999). Relatively thinner walls of smaller vessels render them more susceptible to the effects of NO donors administered to the adventitia (in this case intraventricular or intrathecal) causing them to respond faster, therefore improving cerebral blood flow (Thomas Je Fau - Rosenwasser and Rosenwasser ; Raabe, Zimmermann et al. 2002; Thomas and McGinnis 2002). In addition, the NO molecule will also be able to penetrate the brain tissue and the subarach-

noid clot and the perivascular blood that coats the blood vessels in order to reach the vascular wall and exert its desired effects. This is clinically very relevant, because local administration via intrathecal or intraventricular route is not only effective in reducing the risk of ischemic brain damage, but also minimizes the hypotensive and other undesired side effects of NO donors which are more likely to occur with systemic administration. It's also noted that continuous or repeated administration of SNP is more efficient and produce better outcome than administration of a single dose (Thomas, Rosenwasser et al. 1999). In some cases vasospasm redeveloped a few hours following single dose administration (Thomas, Rosenwasser et al. 1999). This has been explained by the fact that oxyhemoglobin rapidly absorb the NO that is delivered with single dose administration, whereas intermittent or continuous administration will provide constant supply of NO that overwhelms oxyhemoglobin scavenging capacity (Thomas, Rosenwasser et al. 1999). Overall, the findings from nitroprusside studies and those from experimental studies on NO donors for the treatment of vasospasm points towards a key role for microcirculatory dysfunction in the etiology of ischemic brain damage and poor functional outcome after aneurismal subarachnoid hemorrhage. The results of our current studies are in line with these findings.

4.8. Weaknesses of the study

There are some limitations to our current study. Although the study showed evidence of microcirculatory occlusion as early as one hour after the hemorrhage, it's still unclear how soon after subarachnoid hemorrhage these changes start to occur. As the period immediately after subarachnoid hemorrhage has the highest mortality rate (Pobereskin 2001; Macpherson, Lewsey et al. 2011), it will be intriguing to study cerebral circulation immediately after subarachnoid hemorrhage i.e. as close to the moment of the hemorrhage as possible. If it's proven that these changes occur right after the hemorrhage, as we expect, then that will confirm the role of microcirculatory failure in the etiology of post hemorrhagic cerebral ischemia throughout all the stages after subarachnoid hemorrhage. Theoretically, that means that early microthrombosis and microvasospasm represent a target for novel therapeutics techniques that will lower the overall mortality and morbidity from subarachnoid hemorrhage. More importantly, it will provide hope to lower the pre-hospital mortality (the highest mortality group) if any therapy against vasospasm and thrombosis can be delivered to patients before they reach medical care. On the other hand, if raised ICP is the reason behind this high pre-hospital mortality, it will be practically impossible to address and the mortality at this stage will remain high.

The other limitation of the study is that the technique only allows imaging of vessels on the surface of the brain. It's not clear from the study if these changes occur in parenchymal blood vessels. Although we are not able to show it yet, we expect these changes to affect the parenchymal vessels as well. Although it's

not formally and statistically proven in this study, we could notice from some images that perivascular blood follows blood vessels as they leave the surface of the brain heading deep into the parenchyma (**Fig. 5**). As perivascular blood is shown to strongly associate with microthrombosis and microvasospasm on the surface, it's expected that the same occur at the parenchymal level. Most of the studies that reported microthrombosis in patients with aneurismal subarachnoid hemorrhage have shown these microthrombi mainly occur in the deep parts of the brain (Kivisaari, Salonen et al. 2001; Romano, Forteza et al. 2002; Rabinstein, Weigand et al. 2005; Stein, Browne et al. 2006). We are not aware of any clinical study showing vasoconstriction in deep parenchymal blood vessels, but most experimental studies so far describing microthrombosis have shown that microthrombi are accompanied by microvasospasm in most cases (Friedrich, Flores et al. 2010).

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS FOR FUTURE RESEARCH

5.1. Conclusions

This study shows as early as one hour after subarachnoid hemorrhage there are changes in cerebral microcirculation in the form of microvasospasms and microthrombosis that are severe enough and extensive enough to cause cerebral ischemia. The presence of these changes in cerebral blood vessels was shown to strongly correlate with the presence of perivascular blood, suggesting a causative role for blood and blood products that are in direct contact with the adventitia of blood vessels in the development of microvasospasm and microthrombosis after subarachnoid hemorrhage. It's known from the literature that oxyhemoglobin is the most important blood product involved in the etiology of vasospasm. The presence of normal plasma hemoglobin after subarachnoid hemorrhage, along with the strong correlation between microvasospasm and microthrombosis and the presence of perivascular blood, as shown in this study, support the widely accepted theory that perivascular oxyhemoglobin is responsible for the development of post hemorrhagic vasospasm through depletion of nitric oxide from the vessel wall. These findings suggest that microcirculatory failure plays a significant role in the etiology of the early brain injury that occurs after aneurismal subarachnoid hemorrhage and accounts for the high mortality and morbidity associated with the disease. NO depletion from the vessel wall, which is induced by perivascular oxyhemoglobin, is the most likely mechanism responsible for acute microcirculatory failure and the early brain injury that follows and results in high morbidity and mortality after subarachnoid hemorrhage.

5.2. Recommendation for future research

As expected with any experimental study, the findings from this study bring some important questions. Based on the results of this study we can make the following suggestions for future research:

1- Examination of the microcirculation deep in the brain parenchyma after subarachnoid hemorrhage

It will be very interesting to examine the parenchymal microcirculation to see if microvasospasm occurs in parenchymal vessel as well, and to see if perivascular blood follows the cerebral vessels deep in the brain tissue. This can be achieved by using the intravital microscopy model as described here, with the only modification being using two photon imaging modality, instead of the camera we used in this study.

2- Investigation of the therapeutic potentials of NO donors and hemoglobin scavengers

We conclude by supporting the role of adventitial oxyhemoglobin induced NO depletion in the etiology of microvasospasm and microthrombosis which is responsible for cerebral ischemia and early brain injury after subarachnoid hemorrhage. Therefore, in theory, in order to prevent or reverse vasospasm and thrombosis, NO has to be constantly delivered to the vessel wall, or oxyhemoglobin completely removed from perivascular space.

NO donors

NO donors that provide NO to the vessel wall have already been used in preliminary clinical studies, and the results from these are so far encouraging. Larger multicentre clinical trials are needed to test the efficacy of NO donors in reversing vasospasm and improving clinical outcome in patients with aneurismal subarachnoid hemorrhage.

Hemoglobin scavengers

Removal of oxyhemoglobin from perivascular space can, in theory, be achieved either surgically or chemically. However, it's practically impossible to surgical remove all the perivascular blood in cases of aneurismal subarachnoid hemorrhage because blood spread along blood vessels across both hemispheres, and the obvious difficulty of achieving this at microcirculatory level. The other option is to chemically remove oxyhemoglobin from the perivascular space by delivering hemoglobin scavengers such as haptoglobin into the perivascular space. Haptoglobin is a naturally occurring plasma protein predominantly synthesized in the liver as an acute response protein (Zhao, Song et al. 2009). Its main physiological function is to bind and neutralized cell free hemoglobin (Boretti Fs Fau - Buehler, Buehler Pw Fau - D'Agnillo et al. ; Asleh, Guetta et al. 2005; Levy, Asleh et al. 2010). It has been shown that haptoglobin reverses the vascular complications of cell free hemoglobin seen in conditions associated with raised intravascular hemoglobin such as sickle cell disease and cerebral malaria (Boretti Fs Fau - Buehler, Buehler Pw Fau - D'Agnillo et al.). Haptoglobin is also

produced by oligodendroglial cells in the brain where it serve to protect neurons against the cytotoxic effects of hemoglobin through its powerful scavenging effect on cell free hemoglobin (Zhao, Song et al. 2009). Zhao et al. have shown that pharmacological or genetic induction of the synthesis of haptoglobin in the brain reduces the extent of brain damage after intracerebral hemorrhage in animals (Zhao, Song et al. 2009; Zhao, Song et al. 2011).

Therefore, with the proven efficacy of haptoglobin against complications caused by cell free hemoglobin in other diseases, further research can be conducted to test the efficacy of haptoglobin in reducing vasospasm and thrombosis in cases of subarachnoid hemorrhage. In theory, there are two ways by which haptoglobin can be delivered onto the adventitial site of blood vessels, where the oxyhemoglobin is anatomically located: genetically or pharmacological induced overproduction of haptoglobin in the brain tissue, or surgical administration into the CSF circulation through the ventricle or subarachnoid cisterns. However both techniques are likely to have some limitations to overcome in order to produce any desired effects. Surgical administration will have the limitation caused by the fact that CSF circulation is disturbed in subarachnoid hemorrhage, which might hinder the delivery of CSF haptoglobin to areas of the brain affected by vasospasm. Pharmacological induction of overproduction of haptoglobin might be technically more feasible, but is expected to require time before it produces significant increases in haptoglobin levels in the brain tissue that are capable of producing the desired effect. Nonetheless, these foreseen limitations should not prohibit research into the therapeutic potential of

haptoglobin and other hemoglobin scavenger in aneurismal subarachnoid hemorrhage, at least at experimental level.

APPENDICES

Appendix 1

Ethical approval

Royal College of Surgeons in Ireland
The Research Ethics Committee
123 St. Stephens Green, Dublin 2, Ireland.
Tel: +353 1 4022373 Fax: +353 1 4022449 Email: recadmin@rcsi.ie

Dr. David Smith, Acting Chair
Ms. Stephanie O'Connor, Convenor

Royal College of Surgeons in Ireland
Coláiste Ríoga na Máinleá in Éirinn

RCSI

21st October, 2010

Mr Khalid Abubaker,
Department of Neurodegeneration,
Royal College of Surgeons in Ireland,
123 St. Stephen's Green,
Dublin 2.
Ireland

Ethics Reference No:	REC586
Project Title:	Mechanisms of brain damage following subarachnoid haemorrhage
Researchers Name:	Mr Khalid Abubaker
Other Individuals Involved:	Professor Nikolaus Plesnila, RCSI Professor Ciaran Bolger, RCSI

Dear Mr Khalid Abubaker,
Thank you for your well written Research Ethics Committee (REC) application.

We are pleased to advise that ethical approval has been granted by the committee for this study.

This letter provides approval for data collection for the time requested in your application and for an additional 6 months. This is to allow for any unexpected delays in proceeding with data collection. Therefore this research ethics approval will expire on 21st April, 2013.

Where data collection is necessary beyond this point, approval for an extension must be sought from the Research Ethics Committee.

This ethical approval is given on the understanding that:

- All personnel listed in the approved application have read, understand and are thoroughly familiar with all aspects of the study.
- Any person using an animal for scientific purposes is in possession of a valid license from the Department of Health and Children for the project.
- A maximum number of 600 animals (mice) will be used for this study.
- Any significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the REC, and an ethical amendment submitted where appropriate.

We wish you all the best with your research.

Yours sincerely,

PP Ms. Stephanie O'Connor (Convenor)
Dr David Smith (Acting Chair)

2010 RCSI
1810–2010
St. Stephen's Green

Appendix 2 Animal license

An Roinn Sláinte
agus Leanaí
DEPARTMENT OF HEALTH AND CHILDREN

Ref. B100/4341

02/09/2010

Dr Nikolaus Plesnila
Royal College of Surgeons Ireland
123 St Stephens Green
Dublin 2

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005

A Chara

I am to forward herewith a licence which has been granted to you by the Minister for Health and Children to enable you to perform experiments on live animals under the Cruelty to Animals Act, 1876 as amended. Certificates A & B have been noted.

Your licence now expires on the 27th August 2015 and if you require any further licences it will be necessary for you to re-apply before that time. Please allow sufficient time for the application to be processed. Any new experiment which has not been detailed in your application for this licence will require re-application.

Mise le meas

Ciarán Hegarty

Ciarán Hegarty
Environmental Health Unit

An Roinn Sláinte agus Leanaí/Department of Health & Children
Hawkins House Dublin 2 Fón/Tel (01) 635 4000 Ríomhphost/Email info@health.gov.ie
Teach Haicin Baile Átha Cliath 2 Facs/Fax (01) 635 4001 Suíomh Gréasáin/Web www.dohc.ie

Protection of Animals used for Scientific/Medical Experiments

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002

In exercise of the powers conferred on the Minister for Health and Children by section 8 of the Cruelty to Animals Act, 1876 a licence is hereby granted to:-

Dr Nikolaus Plesnila, Royal College of Surgeons Ireland, 123 St Stephens Green, Dublin 2.

Subject to the conditions set out on the reverse, for the performance on living animals of the experiments scheduled beneath, at the following premises:-

Biomedical Research Facility, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2.

General description and objective of the experiments:-
See attached protocol for information.

Particulars of:-

(a) Type of Animal(s)	*Number of each type*
Mice	3020 in total

(b) Individual animal use

Type of animal(s)	*Procedure*	*Frequency and duration of procedure per animal*
Mice	Anaesthesia	Once, 30min
	Intubation and mechanical ventilation	Once, 30min
	Implantation of ICP and Laser-Doppler probes	Once, 10min
	Induction of SAH	Once, 6min
	i.p. application of ^{14}C-iodoantipyrene	Once, 2 sec
	Cervical dislocation in anaesthesia and brain removal	30 seconds later
	Anaesthesia	Once, 2-6 hrs
	Intubation and mechanical ventilation	Once, 10 min
	Establishment of arterial line	Once, 10 min
	Preparation of cranial window	Once, 10 min
	i.v. application of fluorescent dyes	Once, 5 min
	Cervical dislocation in deep isoflurane anaesthesia and brain removal	
	Anaesthesia with Isoflurane N_2O	Once, 10 min
	Opening of the chest and puncture of the left ventricle	
	Opening of the right atrium	Once, terminal
	i.p. injection	2x daily, 10 sec
	Oral gavage	
	Intracerebroventricular (icv) or infusion into the jugular vein	

(c) Type of anaesthetic (if any):- **Midazolam, Medetomidine, Isoflurane, N_2O, Atipamezol, Flumazenil.**

This licence authorises the attached researchers to perform experiments on animals under the supervision and direct responsibility of the above licensee provided that such experiments complies with the conditions set out in this licence.

(d) Unless earlier revoked this licence shall remain in force until the 27th day of August 2015.

Signed on behalf of the Minister for Health and Children

Dated this 1 day of October 2010

Michael Murray

A person authorised in that behalf by the said Minister

Name of researchers

Dr Micaela Gallozzi

Dr Gang Chen

Dr Susanne Schwarzmaier

Dr Lilja Thoenes

Mr Stephen Duff

Mr Khalid Abobaker

D. General Description and Objective of Experiments

[**A detailed protocol must also be submitted**. For diagnostic and forensic work, details of each procedure which the applicant may wish to use must be given. (A monograph or copy of a standard method will suffice)]

Stroke is a clinical condition characterized by neurological dysfunction, e.g. paralysis, inability to speak or understand, coma, or death, due to impaired blood supply in the brain. It can be induced by ischemia (lack of blood supply) or by haemorrhage (bleeding) and is the third leading cause of death and long term disability in industrialized countries. Despite recent advances in the treatment of stroke less than 5% of all stroke patients receive causal treatment, i.e. treatment that prevents brain damage. Therefore there is an urgent need in clarifying the mechanisms by which stroke causes brain damage in order to develop novel treatment options.

Cerebral hemorrhages cause only 20% of all strokes but are responsible for ~50% of the entire stroke-related *mortality*, i.e. *hemorrhagic* stroke is 4.5 times more fatal than ischemic stroke (Rosamond, Folsom et al., 1999). One of the most common and severe causes for hemorrhagic stroke is subarachnoid hemorrhage (SAH), a bleeding from a large extra-parenchymal cerebral vessel mostly caused by a ruptured congenital aneurysm. SAH has an over all mortality of about 50% and over 30% of survivors remain severely disabled (van Gijn and Rinkel, 2001). Despite these facts and some pathophysiological similarities, SAH is by far less frequently investigated than ischemic stroke. Consequently, several important aspects of the pathophysiology of SAH are not well defined thereby hampering the development of novel therapeutic strategies (Cahill and Zhang, 2009).

It is known that cerebral blood flow (CBF) is reduced in the first few hours after SAH and that the resulting cerebral ischemia is the most important predictor for bad functional outcome and death following SAH. Since the cerebral perfusion pressure (CPP) is often normal following SAH and large cerebral vessels show normal flow, the main reason for post-haemorrhagic seems to be on the level of the microcirculation. If this is indeed the case, when and how severe the microcirculation is affected, what mechanisms are responsible for microcirculatory failure following SAH, and if this condition can be tackled therapeutically is, however, largely unknown yet.

Therefore the aim of the current research project is to identify the mechanisms of early post-hemorrhagic CBF reduction and to develop novel therapeutic options for the prevention of SAH-induced brain injury.

Aim 1: Investigate absolute cerebral blood flow following experimental SAH (and sham operation) by ^{14}C-iodoantipyrene autoradiography over time (30s, 3 min, 20 min, 1h, 3h, 6h, 24h, and 72h)

Aim 2: Investigate if increased intracranial pressure is responsible for SAH-induced brain injury. Animals are subjected to decompressive craniectomy (or sham surgery) before, 20 min, 1h, 3h, and 6 h after SAH

Aim 3: Investigate the cerebral microcirculation following experimental SAH by intravital fluorescence microscopy over time (3h, 6h, 12h, 18h, 24h, and 72h)

Aim 4: Investigate whether cerebral vessels react to physiological stimuli (CO_2, adrenaline, vasopressin) following experimental SAH by intravital fluorescence microscopy over time (3h, 6h, 12h, 18h, 24h, and 72h)

Aim 5: Measure nitric oxide production in cerebral endothelial cells following experimental SAH by intravital fluorescence microscopy over time (3h, 6h, 12h, 18h, 24h, and 72h)

Aim 6: Investigate the role of the nitric oxide system for SAH-induced brain injury (neuronal cell death, brain oedema formation, neuronal function, 1h, 3h, 6h, 12h, and 24h)

Aim 7: Measure free haemoglobin in plasma following experimental SAH over time (1 min, 15 min, 30 min, 1h, 3h, 6h, 24h, and 72h)

Aim 8: Investigate how application of free haemoglobin scavengers modulate microvasospasm and brain injury following experimental SAH (before, 1 min, 30 min, 1h, 3h, and 6h)

Aim 9: Investigate the blood plasma, brain tissue, and cerebral vessel proteome following experimental SAH over time (1h, 3h, 6h, 12h, 18h, 24h, 48h, and 72h)

Aim 10: Investigate the role of inflammatory cells and inflammation for SAH-induced brain injury (neuronal cell death, brain oedema formation, neuronal function ; before, 1 min, 30 min, 1h, 3h, and 6h)

Appendix 3

SAH Protocol

Department of Neurodegeneration, RCSI

Dr. Khalid Abubaker, Ref (B100/4299).

SAH protocol

Experiment: _____ Date

Protocol: _____ REC No: 586

Mouse No: _____

Sex: Male Female

Strain: C57 Bl6 TIE2GFP Thy1CFP

Weight: ____ g

Start: ____:____ h End: ____:____ h

Anaesthesia: Isoflurane: concentration % duration sec

Triple combination (I.P) dose: Induction: _____ml maintenance: _____ml

Right temporal *mini-craniotomy* for ICP probe: Start Time: ____:____h End Time: ____:____h

ICP monitoring: Start Time: ____:____h value: SAH Time: ____:____h value: End ____:____h Value

Carotid preparation: start time: ____:____h end time: ____:____h

Right femoral artery catheterization: Start: ____:____h End: ____:____h **ABG sampling: Y/N**

Cranial window:

IVM:

Brain extraction:

Specials:

Signature:

Appendix 4

Protocol for measurement of plasma hemoglobin

Protocol for measurement of plasma hemoglobin

Anesthesia: triple combination

Surgery: supine position, Coronal incision below the diaphragm. Gentle dissection while dissecting skin and opening the peritoneum to avoid bleeding and minimize tissue trauma.

Make a small incision on the right part of the diaphragm. Extend the incision gently on to the left side, care not to cause bleeding by damaging the inferior vena cava or the heart.

Isolate the heart and position it so the right ventricle is clearly seen.

Aspiration of blood: use appropriate needle size needle to avoid hemolysis, (not too big to cause trauma to the heart or too small to cause hemolysis while aspirating). Normally size 23G.

The syringe is coated with heparin by installing one drop of heparin into it and rinsing the whole length of it with this one drop and then aspirating to dryness (So that no drops of heparin left over in the syringe).

Aspirate blood from the heart very gently: avoid turbulence at any stage. Special attention when withdrawing the needle from the heart, make sure there is no negative pressure in the syringe because this will cause turbulence and hence hemolysis.

Aspirate 500 to 600µL of blood in 1 ml syringe pre-coated with heparin as mentioned above.

Remove the needle and gently empty the blood into heparin coated Eppendorf tube.

Pre-coat the Eppendorf tube by filling it with heparin and then aspirate to dryness (so that no heparin drops are left behind).

Centrifugation:

1000 CPM for 10 min+ 1600 CPM for 20 min

Aspirate 50-100 µL of plasma into l000 µL tube

Complete to volume to 1000 µL by adding distilled water

Spectrophotometry:

Get the absorbance at 562, 578 , 598 wavelengths.

Apply the results into this equation: 155XE578-81xE562-69XE598

Multiply the result by the dilution factor

The result is plasma hemoglobin concentration in mg/dl.

Bibliography

Afshar, J. K., R. M. Pluta, et al. (1995). "Effect of intracarotid nitric oxide on primate cerebral vasospasm after subarachnoid hemorrhage." J Neurosurg **83**(1): 118-122.
Agrawal, A., R. Patir, et al. (2009). "Role of intraventricular sodium nitroprusside in vasospasm secondary to aneurysmal subarachnoid haemorrhage: a 5-year prospective study with review of the literature." Minim Invasive Neurosurg **52**(1): 5-8.
Akopov, S., R. Sercombe, et al. (1996). "Cerebrovascular reactivity: role of endothelium/platelet/leukocyte interactions." Cerebrovasc Brain Metab Rev **8**(1): 11-94.
Alaraj, A., F. T. Charbel, et al. (2009). "Peri-operative measures for treatment and prevention of cerebral vasospasm following subarachnoid hemorrhage." Neurol Res **31**(6): 651-659.
Asano, T. "Oxyhemoglobin as the principal cause of cerebral vasospasm: a holistic view of its actions." (0939-0146 (Electronic)).
Asleh, R., J. Guetta, et al. (2005). "Haptoglobin genotype- and diabetes-dependent differences in iron-mediated oxidative stress in vitro and in vivo." Circ Res **96**(4): 435-441.
Barrows, L. J., F. T. Hunter, et al. (1955). "The nature and clinical significance of pigments in the cerebrospinal fluid." Brain **78**(1): 59-80.
Beck, J. and A. Raabe (2011). "Clazosentan: prevention of cerebral vasospasm and the potential to overcome infarction." Acta Neurochir Suppl **110**(Pt 2): 147-150.
Bederson Jb Fau - Levy, A. L., W. H. Levy Al Fau - Ding, et al. "Acute vasoconstriction after subarachnoid hemorrhage." (0148-396X (Print)).
Bederson, J. B., I. M. Germano, et al. (1995). "Cortical blood flow and cerebral perfusion pressure in a new noncraniotomy model of subarachnoid hemorrhage in the rat." Stroke **26**(6): 1086-1091; discussion 1091-1082.
Bederson, J. B., A. L. Levy, et al. (1998). "Acute vasoconstriction after subarachnoid hemorrhage." Neurosurgery **42**(2): 352-360; discussion 360-352.
Boretti Fs Fau - Buehler, P. W., F. Buehler Pw Fau - D'Agnillo, et al. "Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs." (1558-8238 (Electronic)).
Cahill, J., J. W. Calvert, et al. (2006). "Mechanisms of early brain injury after subarachnoid hemorrhage." J Cereb Blood Flow Metab **26**(11): 1341-1353.
Carpenter, D. A., R. L. Grubb, Jr., et al. (1991). "Cerebral oxygen metabolism after aneurysmal subarachnoid hemorrhage." J Cereb Blood Flow Metab **11**(5): 837-844.
Cebral, J. R., F. Mut, et al. (2011). "Quantitative characterization of the hemodynamic environment in ruptured and unruptured brain aneurysms." AJNR Am J Neuroradiol **32**(1): 145-151.
Cebral, J. R., F. Mut, et al. (2011). "Association of hemodynamic characteristics and cerebral aneurysm rupture." AJNR Am J Neuroradiol **32**(2): 264-270.

Cheng, G., L. Wei, et al. (2009). "Atorvastatin ameliorates cerebral vasospasm and early brain injury after subarachnoid hemorrhage and inhibits caspase-dependent apoptosis pathway." BMC Neurosci **10**: 7.

Chou, S. H., E. E. Smith, et al. (2008). "A randomized, double-blind, placebo-controlled pilot study of simvastatin in aneurysmal subarachnoid hemorrhage." Stroke **39**(10): 2891-2893.

Chyatte, D., J. Reilly, et al. (1990). "Morphometric analysis of reticular and elastin fibers in the cerebral arteries of patients with intracranial aneurysms." Neurosurgery **26**(6): 939-943.

Coenen, V. A., C. A. Hansen, et al. (1998). "Endovascular treatment for symptomatic cerebral vasospasm after subarachnoid hemorrhage: transluminal balloon angioplasty compared with intraarterial papaverine." Neurosurg Focus **5**(4): e6.

de la Monte, S. M., G. W. Moore, et al. (1985). "Risk factors for the development and rupture of intracranial berry aneurysms." Am J Med **78**(6 Pt 1): 957-964.

Denton, I. C., J. T. Robertson, et al. (1971). "An assessment of early platelet activity in experimental subarachnoid hemorrhage and middle cerebral artery thrombosis in the cat." Stroke **2**(3): 268-272.

Duff, T. A., J. Louie, et al. (1988). "Erythrocytes are essential for development of cerebral vasculopathy resulting from subarachnoid hemorrhage in cats." Stroke **19**(1): 68-72.

Echlin, F. A. (1965). "Spasm of basilar and vertebral arteries caused by experimental subarachnoid hemorrhage." J Neurosurg **23**(1): 1-11.

Egemen, N., R. K. Turker, et al. (1993). "The effect of intrathecal sodium nitroprusside on severe chronic vasospasm." Neurol Res **15**(5): 310-315.

Feigin, V. L., G. J. Rinkel, et al. (1998). "Calcium antagonists in patients with aneurysmal subarachnoid hemorrhage: a systematic review." Neurology **50**(4): 876-883.

Feiler, S., B. Friedrich, et al. (2010). "Standardized induction of subarachnoid hemorrhage in mice by intracranial pressure monitoring." J Neurosci Methods **190**(2): 164-170.

Ferguson, G. G. (1972). "Physical factors in the initiation, growth, and rupture of human intracranial saccular aneurysms." J Neurosurg **37**(6): 666-677.

Findlay, J. M., R. L. Macdonald, et al. (1991). "Current concepts of pathophysiology and management of cerebral vasospasm following aneurysmal subarachnoid hemorrhage." Cerebrovasc Brain Metab Rev **3**(4): 336-361.

Fisher, C. M., J. P. Kistler, et al. (1980). "Relation of cerebral vasospasm to subarachnoid hemorrhage visualized by computerized tomographic scanning." Neurosurgery **6**(1): 1-9.

Forget, T. R., Jr., R. Benitez, et al. (2001). "A review of size and location of ruptured intracranial aneurysms." Neurosurgery **49**(6): 1322-1325; discussion 1325-1326.

Foutrakis, G. N., H. Yonas, et al. (1999). "Saccular aneurysm formation in curved and bifurcating arteries." AJNR Am J Neuroradiol **20**(7): 1309-1317.

Franks, A. J. (1978). "Prognostic factors in ruptured aneurysms of the circle of Willis: the significance of systemic hypertension." Neuropathol Appl Neurobiol **4**(1): 61-70.

Friedrich, B., F. Muller, et al. (2011). "Experimental subarachnoid hemorrhage causes early and long-lasting microarterial constriction and microthrombosis: an in-vivo microscopy study." J Cereb Blood Flow Metab.

Friedrich, V., R. Flores, et al. (2010). "Luminal platelet aggregates in functional deficits in parenchymal vessels after subarachnoid hemorrhage." Brain Res **1354**: 179-187.

Fujii, Y., S. Takeuchi, et al. (1995). "Hemostasis in spontaneous subarachnoid hemorrhage." Neurosurgery **37**(2): 226-234.

Fujita K Fau - Tamaki, N. and N. Tamaki "[Coagulation abnormalities in stroke--disseminated intravascular coagulation as a complication of subarachnoid hemorrhage]." (0047-1860 (Print)).

Gao, J., H. Wang, et al. (2006). "A novel apoE-derived therapeutic reduces vasospasm and improves outcome in a murine model of subarachnoid hemorrhage." Neurocrit Care **4**(1): 25-31.

Gao, Y., X. S. Ding, et al. (2009). "Neuroprotective effects of edaravone on early brain injury in rats after subarachnoid hemorrhage." Chin Med J (Engl) **122**(16): 1935-1940.

Goretski, J. and T. C. Hollocher (1988). "Trapping of nitric oxide produced during denitrification by extracellular hemoglobin." J Biol Chem **263**(5): 2316-2323.

Greenberg, M. S. M. D. (2006). Handbook of neurosurgery. Lakeland, FL, Greenberg Graphics ; New York : Thieme Medical Publishers.

Grubb, R. L., Jr., M. E. Raichle, et al. (1977). "Effects of subarachnoid hemorrhage on cerebral blood volume, blood flow, and oxygen utilization in humans." J Neurosurg **46**(4): 446-453.

Guix, F. X., I. Uribesalgo, et al. (2005). "The physiology and pathophysiology of nitric oxide in the brain." Prog Neurobiol **76**(2): 126-152.

Haley, E. C., Jr., N. F. Kassell, et al. (1997). "A randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in North America." J Neurosurg **86**(3): 467-474.

Hasan, D., K. W. Lindsay, et al. (1991). "Treatment of acute hydrocephalus after subarachnoid hemorrhage with serial lumbar puncture." Stroke **22**(2): 190-194.

Hauerberg J Fau - Eskesen, V., J. Eskesen V Fau - Rosenorn, et al. "The prognostic significance of intracerebral haematoma as shown on CT scanning after aneurysmal subarachnoid haemorrhage." (0268-8697 (Print)).

Hegedus, K. "Some observations on reticular fibers in the media of the major cerebral arteries. A comparative study of patients without vascular diseases and those with ruptured berry aneurysms." (0090-3019 (Print)).

Heuer Gg Fau - Smith, M. J., J. P. Smith Mj Fau - Elliott, et al. "Relationship between intracranial pressure and other clinical variables in patients with aneurysmal subarachnoid hemorrhage." (0022-3085 (Print)).

Honma, Y., B. R. Clower, et al. (1989). "Comparison of intimal platelet accumulation in cerebral arteries in two experimental models of subarachnoid hemorrhage." Neurosurgery **24**(4): 487-490.

Hop, J. W., G. J. Rinkel, et al. (1997). "Case-fatality rates and functional outcome after subarachnoid hemorrhage: a systematic review." Stroke **28**(3): 660-664.

Ignarro, L. J. (1990). "Biosynthesis and metabolism of endothelium-derived nitric oxide." Annu Rev Pharmacol Toxicol **30**: 535-560.

Ilveskero S Fau - Juvela, S., J. Juvela S Fau - Siironen, et al. "D-dimer predicts outcome after aneurysmal subarachnoid hemorrhage: no effect of thromboprophylaxis on coagulation activity." (1524-4040 (Electronic)).

Itoyama, Y., S. Fujioka, et al. (1994). "Significance of elevated thrombin-antithrombin III complex and plasmin-alpha 2-plasmin inhibitor complex in the acute stage of nontraumatic subarachnoid hemorrhage." Neurosurgery **35**(6): 1055-1060.

Jakobsen M Fau - Overgaard, J., E. Overgaard J Fau - Marcussen, et al. "Relation between angiographic cerebral vasospasm and regional CBF in patients with SAH." (0001-6314 (Print)).

Jan, M., F. Buchheit, et al. (1988). "Therapeutic trial of intravenous nimodipine in patients with established cerebral vasospasm after rupture of intracranial aneurysms." Neurosurgery **23**(2): 154-157.

Jang, Y. G., D. Ilodigwe, et al. (2009). "Metaanalysis of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage." Neurocrit Care **10**(1): 141-147.

Jung Cs Fau - Iuliano, B. A., J. Iuliano Ba Fau - Harvey-White, et al. "Association between cerebrospinal fluid levels of asymmetric dimethyl-L-arginine, an endogenous inhibitor of endothelial nitric oxide synthase, and cerebral vasospasm in a primate model of subarachnoid hemorrhage." (0022-3085 (Print)).

Jung, C. S., E. H. Oldfield, et al. (2007). "Association of an endogenous inhibitor of nitric oxide synthase with cerebral vasospasm in patients with aneurysmal subarachnoid hemorrhage." J Neurosurg **107**(5): 945-950.

Juvela, S. (1990). "Cerebral infarction and release of platelet thromboxane after subarachnoid hemorrhage." Neurosurgery **27**(6): 929-935.

Juvela S Fau - Siironen, J. and J. Siironen "D-dimer as an independent predictor for poor outcome after aneurysmal subarachnoid hemorrhage." (1524-4628 (Electronic)).

Juvela, S., M. Hillbom, et al. (1991). "Platelet thromboxane release and delayed cerebral ischemia in patients with subarachnoid hemorrhage." J Neurosurg **74**(3): 386-392.

Juvela, S., M. Kaste, et al. (1990). "Effect of nimodipine on platelet function in patients with subarachnoid hemorrhage." Stroke **21**(9): 1283-1288.
Juvela, S., M. Kaste, et al. (1990). "Platelet thromboxane release after subarachnoid hemorrhage and surgery." Stroke **21**(4): 566-571.
Juvela, S., J. Ohman, et al. (1991). "Angiographic vasospasm and release of platelet thromboxane after subarachnoid hemorrhage." Stroke **22**(4): 451-455.
Juvela, S., M. Porras, et al. (2008). "Natural history of unruptured intracranial aneurysms: probability of and risk factors for aneurysm rupture." J Neurosurg **108**(5): 1052-1060.
Kahn, S. E., B. F. Watkins, et al. (1981). "An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin." Ann Clin Lab Sci **11**(2): 126-131.
Kajikawa H Fau - Ohta, T., Y. Ohta T Fau - Yoshikawa, et al. "Cerebral vasospasm and hemoglobins--clinical and experimental studies." (0470-8105 (Print)).
Kamii, H., I. Kato, et al. (1999). "Amelioration of vasospasm after subarachnoid hemorrhage in transgenic mice overexpressing CuZn-superoxide dismutase." Stroke **30**(4): 867-871; discussion 872.
Kassell, N. F., E. C. Haley, Jr., et al. (1996). "Randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in Europe, Australia, and New Zealand." J Neurosurg **84**(2): 221-228.
Kasuya, H., B. K. Weir, et al. (1993). "Mechanism of oxyhemoglobin-induced release of endothelin-1 from cultured vascular endothelial cells and smooth-muscle cells." J Neurosurg **79**(6): 892-898.
Kataoka, H., S. W. Kim, et al. (2004). "Leukocyte-endothelium interactions during permanent focal cerebral ischemia in mice." J Cereb Blood Flow Metab **24**(6): 668-676.
Kelly, P. J., R. J. Gorten, et al. (1977). "Cerebral perfusion, vascular spasm, and outcome in patients with ruptured intracranial aneurysms." J Neurosurg **47**(1): 44-49.
Kim, C., J. Cervos-Navarro, et al. (1993). "Degenerative changes in the internal elastic lamina relating to the development of saccular cerebral aneurysms in rats." Acta Neurochir (Wien) **121**(1-2): 76-81.
Kistler, J. P., R. M. Crowell, et al. (1983). "The relation of cerebral vasospasm to the extent and location of subarachnoid blood visualized by CT scan: a prospective study." Neurology **33**(4): 424-436.
Kivisaari, R. P., O. Salonen, et al. (2001). "MR imaging after aneurysmal subarachnoid hemorrhage and surgery: a long-term follow-up study." AJNR Am J Neuroradiol **22**(6): 1143-1148.
Kopera, M., H. Majchrzak, et al. (1999). "[Prognostic factors in patients with intracerebral hematoma caused by ruptured middle cerebral artery aneurysm]." Neurol Neurochir Pol **33**(2): 389-401.

Kulcsar, Z., A. Ugron, et al. (2011). "Hemodynamics of cerebral aneurysm initiation: the role of wall shear stress and spatial wall shear stress gradient." AJNR Am J Neuroradiol **32**(3): 587-594.

Kusanagi H Fau - Teramoto, A., T. Teramoto A Fau - Shimura, et al. "[The relationship between the severity of a neurological condition and the blood coagulation/fibrinolytic system in patients with spontaneous subarachnoid hemorrhage]." (0301-2603 (Print)).

Lanzino, G. and N. F. Kassell (1999). "Double-blind, randomized, vehicle-controlled study of high-dose tirilazad mesylate in women with aneurysmal subarachnoid hemorrhage. Part II. A cooperative study in North America." J Neurosurg **90**(6): 1018-1024.

Lanzino, G., N. F. Kassell, et al. (1999). "Double-blind, randomized, vehicle-controlled study of high-dose tirilazad mesylate in women with aneurysmal subarachnoid hemorrhage. Part I. A cooperative study in Europe, Australia, New Zealand, and South Africa." J Neurosurg **90**(6): 1011-1017.

Levy, A. P., R. Asleh, et al. (2010). "Haptoglobin: basic and clinical aspects." Antioxid Redox Signal **12**(2): 293-304.

Linn, F. H., G. J. Rinkel, et al. (1996). "Incidence of subarachnoid hemorrhage: role of region, year, and rate of computed tomography: a meta-analysis." Stroke **27**(4): 625-629.

Lynch, J. R., H. Wang, et al. (2005). "Simvastatin reduces vasospasm after aneurysmal subarachnoid hemorrhage: results of a pilot randomized clinical trial." Stroke **36**(9): 2024-2026.

Macdonald, R. L., R. T. Higashida, et al. (2011). "Clazosentan, an endothelin receptor antagonist, in patients with aneurysmal subarachnoid haemorrhage undergoing surgical clipping: a randomised, double-blind, placebo-controlled phase 3 trial (CONSCIOUS-2)." Lancet Neurol **10**(7): 618-625.

Macdonald, R. L., N. F. Kassell, et al. (2008). "Clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage (CONSCIOUS-1): randomized, double-blind, placebo-controlled phase 2 dose-finding trial." Stroke **39**(11): 3015-3021.

Macdonald, R. L., R. M. Pluta, et al. (2007). "Cerebral vasospasm after subarachnoid hemorrhage: the emerging revolution." Nat Clin Pract Neurol **3**(5): 256-263.

Macdonald, R. L. and B. K. Weir (1991). "A review of hemoglobin and the pathogenesis of cerebral vasospasm." Stroke **22**(8): 971-982.

Macdonald, R. L., B. K. Weir, et al. (1991). "Etiology of cerebral vasospasm in primates." J Neurosurg **75**(3): 415-424.

Macpherson, K. J., J. D. Lewsey, et al. (2011). "Trends in incidence and in short term survival following a subarachnoid haemorrhage in Scotland, 1986 - 2005: a retrospective cohort study." BMC Neurol **11**: 38.

Martin, W., G. M. Villani, et al. (1985). "Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and

by methylene blue in the rabbit aorta." J Pharmacol Exp Ther **232**(3): 708-716.
Mayberg, M. R., T. Okada, et al. (1990). "The role of hemoglobin in arterial narrowing after subarachnoid hemorrhage." J Neurosurg **72**(4): 634-640.
Mayfrank, L., B. O. Hutter, et al. (2001). "Influence of intraventricular hemorrhage on outcome after rupture of intracranial aneurysm." Neurosurg Rev **24**(4): 185-191.
McGirt, M. J., A. Parra, et al. (2002). "Attenuation of cerebral vasospasm after subarachnoid hemorrhage in mice overexpressing extracellular superoxide dismutase." Stroke **33**(9): 2317-2323.
Misra, H. P. and I. Fridovich (1972). "The generation of superoxide radical during the autoxidation of hemoglobin." J Biol Chem **247**(21): 6960-6962.
Mohr, G., G. Ferguson, et al. (1983). "Intraventricular hemorrhage from ruptured aneurysm. Retrospective analysis of 91 cases." J Neurosurg **58**(4): 482-487.
Momin, E. N., K. E. Schwab, et al. (2009). "Controlled delivery of nitric oxide inhibits leukocyte migration and prevents vasospasm in haptoglobin 2-2 mice after subarachnoid hemorrhage." Neurosurgery **65**(5): 937-945; discussion 945.
Morga, R., R. Czepko, et al. (2007). "Assessment of the haemostatic system in patients surgically treated for ruptured cerebral aneurysm." Neurol Neurochir Pol **41**(4): 296-305.
Munakata, A., H. Ohkuma, et al. (2011). "Effect of a free radical scavenger, edaravone, on free radical reactions: related signal transduction and cerebral vasospasm in the rabbit subarachnoid hemorrhage model." Acta Neurochir Suppl **110**(Pt 2): 17-22.
Murai, Y., S. Kominami, et al. (2005). "The long-term effects of transluminal balloon angioplasty for vasospasms after subarachnoid hemorrhage: analyses of cerebral blood flow and reactivity." Surg Neurol **64**(2): 122-126; discussion 127.
Nakagawa T Fau - Suga, S., K. Suga S Fau - Mayanagi, et al. "Predicting the overall management outcome in patients with a subarachnoid hemorrhage accompanied by a massive intracerebral or full-packed intraventricular hemorrhage: a 15-year retrospective study." (0090-3019 (Print)).
Nieuwkamp, D. J., L. E. Setz, et al. (2009). "Changes in case fatality of aneurysmal subarachnoid haemorrhage over time, according to age, sex, and region: a meta-analysis." Lancet Neurol **8**(7): 635-642.
Nina, P., G. Schisano, et al. (2001). "A study of blood coagulation and fibrinolytic system in spontaneous subarachnoid hemorrhage. Correlation with hunt-hess grade and outcome." Surg Neurol **55**(4): 197-203.
Nixon, A. M., M. Gunel, et al. (2010). "The critical role of hemodynamics in the development of cerebral vascular disease." J Neurosurg **112**(6): 1240-1253.

Nozaki, K., M. A. Moskowitz, et al. (1993). "Possible origins and distribution of immunoreactive nitric oxide synthase-containing nerve fibers in cerebral arteries." J Cereb Blood Flow Metab **13**(1): 70-79.
Nozaki, K., S. Okamoto, et al. (1990). "Red blood cells are essential for late vasospasm following experimentally induced subarachnoid hemorrhage in dogs." Neurol Med Chir (Tokyo) **30**(1): 10-15.
Ohkuma, H., K. Ogane, et al. (1993). "Impairment of anti-platelet-aggregating activity of endothelial cells after experimental subarachnoid hemorrhage." Stroke **24**(10): 1541-1545; discussion 1545-1546.
Ohkuma, H., S. Suzuki, et al. (1990). "[Disturbance of inhibitory capacity of endothelial cell for platelet adhesion or aggregation following experimental subarachnoid hemorrhage]." No Shinkei Geka **18**(1): 47-52.
Okada, Y., B. R. Copeland, et al. (1994). "P-selectin and intercellular adhesion molecule-1 expression after focal brain ischemia and reperfusion." Stroke **25**(1): 202-211.
Okuyama T Fau - Sasamori, Y., H. Sasamori Y Fau - Takahashi, et al. "[Study of multiple cerebral aneurysms comprised of both ruptured and unruptured aneurysm--an analysis of incidence rate with respect to site and size]." (0301-2603 (Print)).
Ostergaard, J. R. (1991). "Aetiology of intracranial saccular aneurysms in childhood." Br J Neurosurg **5**(6): 575-580.
Ostergaard, J. R., E. Reske-Nielsen, et al. (1989). "Deficiency of reticular fibers in cerebral arteries. On the etiology of saccular aneurysms in childhood." Br J Neurosurg **3**(1): 113-115.
Ostrowski, R. P., A. R. Colohan, et al. (2006). "Molecular mechanisms of early brain injury after subarachnoid hemorrhage." Neurol Res **28**(4): 399-414.
Park, S., M. Yamaguchi, et al. (2004). "Neurovascular protection reduces early brain injury after subarachnoid hemorrhage." Stroke **35**(10): 2412-2417.
Pathak, A., S. N. Mathuriya, et al. (2003). "Intermittent low dose intrathecal sodium nitroprusside therapy for treatment of symptomatic aneurysmal SAH-induced vasospasm." Br J Neurosurg **17**(4): 306-310.
Peltonen, S., S. Juvela, et al. (1997). "Hemostasis and fibrinolysis activation after subarachnoid hemorrhage." J Neurosurg **87**(2): 207-214.
Peterson, J. W., L. Roussos, et al. (1990). "Evidence of the role of hemolysis in experimental cerebral vasospasm." J Neurosurg **72**(5): 775-781.
Petruk, K. C., M. West, et al. (1988). "Nimodipine treatment in poor-grade aneurysm patients. Results of a multicenter double-blind placebo-controlled trial." J Neurosurg **68**(4): 505-517.
Philippon, J., R. Grob, et al. (1986). "Prevention of vasospasm in subarachnoid haemorrhage. A controlled study with nimodipine." Acta Neurochir (Wien) **82**(3-4): 110-114.
Pisapia, J. M., X. Xu, et al. (2011). "Microthrombosis after experimental subarachnoid hemorrhage: Time course and effect of red blood cell-bound thrombin-activated pro-urokinase and clazosentan." Exp Neurol.
Pluta, R. M. "Dysfunction of nitric oxide synthases as a cause and therapeutic target in delayed cerebral vasospasm after SAH." (0161-6412 (Print)).

Pluta, R. M. (2005). "Delayed cerebral vasospasm and nitric oxide: review, new hypothesis, and proposed treatment." Pharmacol Ther **105**(1): 23-56.
Pluta, R. M., J. K. Afshar, et al. (1998). "Temporal changes in perivascular concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin after subarachnoid hemorrhage." J Neurosurg **88**(3): 557-561.
Pluta, R. M., A. Dejam, et al. (2005). "Nitrite infusions to prevent delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage." JAMA **293**(12): 1477-1484.
Pluta Rm Fau - Boock, R. J., J. K. Boock Rj Fau - Afshar, et al. "Source and cause of endothelin-1 release into cerebrospinal fluid after subarachnoid hemorrhage." (0022-3085 (Print)).
Pluta, R. M., E. H. Oldfield, et al. (1997). "Reversal and prevention of cerebral vasospasm by intracarotid infusions of nitric oxide donors in a primate model of subarachnoid hemorrhage." J Neurosurg **87**(5): 746-751.
Pluta, R. M., B. G. Thompson, et al. (1996). "Loss of nitric oxide synthase immunoreactivity in cerebral vasospasm." J Neurosurg **84**(4): 648-654.
Pobereskin, L. H. (2001). "Incidence and outcome of subarachnoid haemorrhage: a retrospective population based study." J Neurol Neurosurg Psychiatry **70**(3): 340-343.
Polin, R. S., V. A. Coenen, et al. (2000). "Efficacy of transluminal angioplasty for the management of symptomatic cerebral vasospasm following aneurysmal subarachnoid hemorrhage." J Neurosurg **92**(2): 284-290.
Prunell, G. F., T. Mathiesen, et al. (2003). "Experimental subarachnoid hemorrhage: subarachnoid blood volume, mortality rate, neuronal death, cerebral blood flow, and perfusion pressure in three different rat models." Neurosurgery **52**(1): 165-175; discussion 175-166.
Raabe, A., M. Zimmermann, et al. (2002). "Effect of intraventricular sodium nitroprusside on cerebral hemodynamics and oxygenation in poor-grade aneurysm patients with severe, medically refractory vasospasm." Neurosurgery **50**(5): 1006-1013; discussion 1013-1004.
Rabinstein, A. A., S. Weigand, et al. (2005). "Patterns of cerebral infarction in aneurysmal subarachnoid hemorrhage." Stroke **36**(5): 992-997.
Rinkel, G. J., M. Djibuti, et al. (1998). "Prevalence and risk of rupture of intracranial aneurysms: a systematic review." Stroke **29**(1): 251-256.
Roach, M. R., S. Scott, et al. (1972). "The hemodynamic importance of the geometry of bifurcations in the circle of Willis (glass model studies)." Stroke **3**(3): 255-267.
Romano, J. G., A. M. Forteza, et al. (2002). "Detection of microemboli by transcranial Doppler ultrasonography in aneurysmal subarachnoid hemorrhage." Neurosurgery **50**(5): 1026-1030; discussion 1030-1021.
Rosengart, A. J., K. E. Schultheiss, et al. (2007). "Prognostic factors for outcome in patients with aneurysmal subarachnoid hemorrhage." Stroke **38**(8): 2315-2321.
Scanarini, M., S. Mingrino, et al. (1978). "Histological and ultrastructural study of intracranial saccular aneurysmal wall." Acta Neurochir (Wien) **43**(3-4): 171-182.

Schubert, G. A., M. Seiz, et al. (2009). "Acute hypoperfusion immediately after subarachnoid hemorrhage: a xenon contrast-enhanced CT study." J Neurotrauma **26**(12): 2225-2231.
Schubert, G. A., M. Seiz, et al. (2011). "Hypoperfusion in the acute phase of subarachnoid hemorrhage." Acta Neurochir Suppl **110**(Pt 1): 35-38.
Schwarzmaier, S. M., S. W. Kim, et al. (2010). "Temporal profile of thrombogenesis in the cerebral microcirculation after traumatic brain injury in mice." J Neurotrauma **27**(1): 121-130.
Sehba, F. A. and J. B. Bederson (2011). "Nitric oxide in early brain injury after subarachnoid hemorrhage." Acta Neurochir Suppl **110**(Pt 1): 99-103.
Sehba, F. A., W. H. Ding, et al. (1999). "Effects of S-nitrosoglutathione on acute vasoconstriction and glutamate release after subarachnoid hemorrhage." Stroke **30**(9): 1955-1961.
Sehba, F. A., G. Mostafa, et al. (2005). "Acute microvascular platelet aggregation after subarachnoid hemorrhage." J Neurosurg **102**(6): 1094-1100.
Sehba, F. A., R. M. Pluta, et al. (2011). "Metamorphosis of subarachnoid hemorrhage research: from delayed vasospasm to early brain injury." Mol Neurobiol **43**(1): 27-40.
Sehba, F. A., A. Y. Schwartz, et al. (2000). "Acute decrease in cerebral nitric oxide levels after subarachnoid hemorrhage." J Cereb Blood Flow Metab **20**(3): 604-611.
Seibert, B., R. P. Tummala, et al. (2011). "Intracranial aneurysms: review of current treatment options and outcomes." Front Neurol **2**: 45.
Seifert, V., B. M. Loffler, et al. (1995). "Endothelin concentrations in patients with aneurysmal subarachnoid hemorrhage. Correlation with cerebral vasospasm, delayed ischemic neurological deficits, and volume of hematoma." J Neurosurg **82**(1): 55-62.
Stein, S. C., K. D. Browne, et al. (2006). "Thromboembolism and delayed cerebral ischemia after subarachnoid hemorrhage: an autopsy study." Neurosurgery **59**(4): 781-787; discussion 787-788.
Suarez-Rivera, O. (1998). "Acute hydrocephalus after subarachnoid hemorrhage." Surg Neurol **49**(5): 563-565.
Suzuki, H., M. Muramatsu, et al. (2003). "Intracranial heme metabolism and cerebral vasospasm after aneurysmal subarachnoid hemorrhage." Stroke **34**(12): 2796-2800.
Thal, S. C. and N. Plesnila (2007). "Non-invasive intraoperative monitoring of blood pressure and arterial pCO2 during surgical anesthesia in mice." J Neurosci Methods **159**(2): 261-267.
Thomas Je Fau - Rosenwasser, R. H. and R. H. Rosenwasser "Reversal of severe cerebral vasospasm in three patients after aneurysmal subarachnoid hemorrhage: initial observations regarding the use of intraventricular sodium nitroprusside in humans." (0148-396X (Print)).
Thomas, J. E. and G. McGinnis (2002). "Safety of intraventricular sodium nitroprusside and thiosulfate for the treatment of cerebral vasospasm in the intensive care unit setting." Stroke **33**(2): 486-492.

Thomas, J. E., A. Nemirovsky, et al. (1997). "Rapid reversal of endothelin-1-induced cerebral vasoconstriction by intrathecal administration of nitric oxide donors." Neurosurgery **40**(6): 1245-1249.
Thomas, J. E., R. H. Rosenwasser, et al. (1999). "Safety of intrathecal sodium nitroprusside for the treatment and prevention of refractory cerebral vasospasm and ischemia in humans." Stroke **30**(7): 1409-1416.
Toda, N. (1990). "Mechanisms of contracting action of oxyhemoglobin in isolated monkey and dog cerebral arteries." Am J Physiol **258**(1 Pt 2): H57-63.
Toda, N. and T. Okamura (1990). "Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle." Biochem Biophys Res Commun **170**(1): 308-313.
Toda, N. and T. Okamura (1991). "Role of nitric oxide in neurally induced cerebroarterial relaxation." J Pharmacol Exp Ther **258**(3): 1027-1032.
Tokuda, Y., T. Inagawa, et al. (1995). "Intracerebral hematoma in patients with ruptured cerebral aneurysms." Surg Neurol **43**(3): 272-277.
Torok, E., M. Klopotowski, et al. (2009). "Mild hypothermia (33 degrees C) reduces intracranial hypertension and improves functional outcome after subarachnoid hemorrhage in rats." Neurosurgery **65**(2): 352-359; discussion 359.
Tseng, M. Y., M. Czosnyka, et al. (2005). "Effects of acute treatment with pravastatin on cerebral vasospasm, autoregulation, and delayed ischemic deficits after aneurysmal subarachnoid hemorrhage: a phase II randomized placebo-controlled trial." Stroke **36**(8): 1627-1632.
Tseng, M. Y., P. J. Hutchinson, et al. (2007). "Effects of acute pravastatin treatment on intensity of rescue therapy, length of inpatient stay, and 6-month outcome in patients after aneurysmal subarachnoid hemorrhage." Stroke **38**(5): 1545-1550.
Turner, C. L., S. Tebbs, et al. (2001). "The influence of hemodynamic stress factors on intracranial aneurysm formation." J Neurosurg **95**(5): 764-770.
Uhl, E., J. Lehmberg, et al. (2003). "Intraoperative detection of early microvasospasm in patients with subarachnoid hemorrhage by using orthogonal polarization spectral imaging." Neurosurgery 52(6): 1307-1315; disacussion 1315-1307.
Vajkoczy, P., B. Meyer, et al. (2005). "Clazosentan (AXV-034343), a selective endothelin A receptor antagonist, in the prevention of cerebral vasospasm following severe aneurysmal subarachnoid hemorrhage: results of a randomized, double-blind, placebo-controlled, multicenter phase IIa study." J Neurosurg **103**(1): 9-17.
van Gijn J Fau - Kerr, R. S., G. J. E. Kerr Rs Fau - Rinkel, et al. "Subarachnoid haemorrhage." (1474-547X (Electronic)).
Vergouwen, M. D., R. J. de Haan, et al. (2010). "Effect of statin treatment on vasospasm, delayed cerebral ischemia, and functional outcome in patients with aneurysmal subarachnoid hemorrhage: a systematic review and meta-analysis update." Stroke **41**(1): e47-52.
Vergouwen, M. D., M. Vermeulen, et al. (2008). "Microthrombosis after aneurysmal subarachnoid hemorrhage: an additional explanation for

delayed cerebral ischemia." J Cereb Blood Flow Metab 28(11): 1761-1770.
Wardlaw, J. M. and P. M. White (2000). "The detection and management of unruptured intracranial aneurysms." Brain 123 (Pt 2): 205-221.
Weir Bk Fau - Kongable, G. L., N. F. Kongable Gl Fau - Kassell, et al. "Cigarette smoking as a cause of aneurysmal subarachnoid hemorrhage and risk for vasospasm: a report of the Cooperative Aneurysm Study." (0022-3085 (Print)).
Westermaier T Fau - Jauss, A., J. Jauss A Fau - Eriskat, et al. "Acute vasoconstriction: decrease and recovery of cerebral blood flow after various intensities of experimental subarachnoid hemorrhage in rats." (0022-3085 (Print)).
Westermaier, T., A. Jauss, et al. (2009). "Time-course of cerebral perfusion and tissue oxygenation in the first 6 h after experimental subarachnoid hemorrhage in rats." J Cereb Blood Flow Metab 29(4): 771-779.
White, P. M. and J. Wardlaw (2003). "Unruptured intracranial aneurysms: prospective data have arrived." Lancet 362(9378): 90-91.
White, R. P., A. A. Hagen, et al. (1975). "Experimental study on the genesis of cerebral vasospasm." Stroke 6(1): 52-57.
Wilkinson, I. M. (1972). "The vertebral artery. Extracranial and intracranial structure." Arch Neurol 27(5): 392-396.
Yamamoto, Y., B. R. Clower, et al. (1991). "Adventitial red blood cells produce intimal platelet accumulation in cerebral arteries of cats following subarachnoid hemorrhage." Stroke 22(3): 373-377.
Yamamoto, Y., B. R. Clower, et al. (1991). "Effect of tissue plasminogen activator on intimal platelet accumulation in cerebral arteries after subarachnoid hemorrhage in cats." Stroke 22(6): 780-784.
Yong-Zhong, G. and H. A. van Alphen (1990). "Pathogenesis and histopathology of saccular aneurysms: review of the literature." Neurol Res 12(4): 249-255.
Zervas, N. T., A. Kuwayama, et al. (1973). "Cerebral arterial spasm. Modification by inhibition of platelet function." Arch Neurol 28(6): 400-404.
Zhang Zd Fau - Yamini, B., T. Yamini B Fau - Komuro, et al. "Vasospasm in monkeys resolves because of loss of and encasement of subarachnoid blood clot." (1524-4628 (Electronic)).
Zhao, X., S. Song, et al. (2009). "Neuroprotective role of haptoglobin after intracerebral hemorrhage." J Neurosci 29(50): 15819-15827.
Zhao, X., S. Song, et al. (2011). "Cytoprotective role of haptoglobin in brain after experimental intracerebral hemorrhage." Acta Neurochir Suppl 111: 107-112.
Zimmermann, M. and V. Seifert (1998). "Endothelin and subarachnoid hemorrhage: an overview." Neurosurgery 43(4): 863-875; discussion 875-866.